The effect of a potential protein binder on ruminal and post-ruminal protein digestion responses

by

Abraham Johannes Hendrik Burger

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Supervisor: Prof CW Cruywagen

Co-supervisor: Dr JHC Van Zyl

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DECLARATION

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ABSTRACT

Title	:	The effect of a potential protein binder on ruminal and post-ruminal protein digestion responses
Name	:	Abraham Johannes Hendrik Burger
Supervisor	:	Prof. C.W. Cruywagen
Institute	:	Department of Animal Sciences, Stellenbosch University
Degree	:	MScAgric (Animal Science)

The objectives of this study were to determine the effect of a potential protein binder on *in sacco* and *in vitro* protein disappearance parameters, protein solubility, degradability of soluble protein and intestinal protein digestibility. Soybean, sunflower and canola oilcake meal (OCM) were the three protein feedstuffs used in the trials as they are commonly included in dairy cattle diets in South Africa. Substrates in all trials were treated with Bioprotect® at a rate equivalent to 0.5 L per tonne for each 1% CP (crude protein) in the substrate. Distilled water was used as the control treatment and was applied at the same rates. Ruminally cannulated lactating Holstein cows were used in an *in sacco* trial and were also used as rumen liquid donors for the *in vitro* trials.

In the first trial, the *in sacco* procedure was used to determine degradation kinetic parameters over time. All three OCMs were used and rumen incubation was done in four cows. Incubation times were 0, 2, 4, 8, 16, 24 and 48 hours. For the 0 h values, samples were water washed and not incubated in the rumen. Dry matter and CP degradation data were fitted to a non-linear model to determine kinetic parameters and effective degradability. The protein binder did not increase resistance against microbial degradation. The 0 h values were increased after Bioprotect® treatment, resulting in higher model-derived a-values, which also resulted in higher effective degradability values for all three substrates compared to the control treatment.

In the second trial, a Daisy^{II} incubator (ANKOM Technology, New York) was used for the *in vitro* incubations. The same three OCMs were used as in Trial 1 and incubation times were also similar. Treatment * time interactions indicated that the protein binder reduced CP degradation of canola meal after 8 h (49.3 vs. 54.2% for Control; P = 0.022) and 16 h of incubation (63.3 vs. 67.5% for Control; P = 0.04). For soybean meal, treatment also tended (P = 0.06) to reduce 16 h CP degradability (72.6 vs. 77.9% for Control). It seems that

Bioprotect® appears to increase CP solubility; however the effect of treatment on CP degradability was not conclusive because of different tendencies observed in the *in sacco* and *in vitro* trials. However, the difference between treatments in the magnitude of *in vitro* CP degradability observed from 4 to 16 h suggested that Bioprotect® may indeed have a reducing effect on the degradability of the potentially degradable fraction but this effect may be shadowed by the increase in the soluble fraction observed in the Bioprotect® treatment.

The third trial was done to determine intestinal protein digestibility using the Ross assay. All three OCMs were used and rumen liquid was collected from six lactating Holstein cows. The first phase of the assay represented a 12 h rumen incubation, which was followed by a one-hour gastric digestion phase and finally, a 24 h intestinal digestion phase. Results showed no difference in protein degradability between treatments.

The fourth trial investigated the effect of Bioprotect® treatment on the solubility of the substrate proteins. Each OCM with and without Bioprotect treatement was incubated in a borate-phosphate buffer for one hour. Samples were analysed for N only. Bioprotect® treatment increased protein solubility and the soluble protein content of soybean oilcake but had no effect on the other oil cakes. The soluble protein content of soybean oil cake was 20.2% for the Bioprotect® treatment vs. 13.1% for the Control ($P < 0.001$).

The fifth trial determined the degradability of the soluble protein in soybean OCM. This was because it was the only substrate where the soluble protein content differed between treatments. After solubilising the protein using the same protocol as in Trial 4, the supernatant was added to a buffered rumen liquid incubation medium similar to that of the *in vitro* trial above. Samples were incubated at 39°C for 0, 2, 8 and 24 hours, followed by N analysis. Bioprotect® significantly reduced the rate and extent of the soluble protein degradation. Over the entire 0 – 24 h incubation period, the mean degradation rate (k_d) was $0.028/h^{-1}$ for the Bioprotect® treatment and $0.036/h^{-1}$ for the Control treatment, clearly demonstrating the depressing effect of Bioprotect® on soluble protein degradation.

The protein binder, Bioprotect®, seem to have some potential to reduce ruminal CP degradability in some OCMs as the degradation of soluble protein in soybean oil cake meal was clearly decreased by Bioprotect®. This may warrant further research as soybean oil cake is a major protein sources in dairy cattle. Further research with lactating dairy cows is warranted to investigate the effect of Bioprotect® on milk production response, especially when lowering the CP content of the diet.

UITTREKSEL

Titel	:	Die invloed van ‘n potensiële proteïenbinder op ruminale en post-ruminale proteïenverteringsresponse
Kandidaat	:	Abraham Johannes Hendrik Burger
Studieleier	:	Prof C.W. Cruywagen
Instansie	:	Departement Veekundige Wetenskappe. Universiteit van Stellenbosch
Graad	:	MScAgric (Veekunde)

Die doel van hierdie studie was om die invloed van ‘n potensiële proteïenbinder op *in sacco* en *in vitro* proteïen-verdwyningsparameters, oplosbaarheid van proteïen, degradeerbaarheid van oplosbare proteïen en intestinale proteïenverteerbaarheid te bepaal. Drie proteïen-grondstowwe wat algemeen in melkbeesdiëte in Suid-Afrika ingesluit word, naamlik soja-, sonneblom- en kanola-oliekoekmeel, is in die proewe gebruik. Bioprotect® is as proteïen-binder gebruik en in al die proewe is die substrate met Bioprotect® óf gedistilleerde water (kontrole) behandel deur dit op die onderskeie substrate te spuit teen ‘n peil ekwivalent aan 0.5 L per ton vir elke 1% RP (ruproteïen). Rumen-gekannuleerde lakterende Holsteinkoeie is in ‘n *in sacco* proef gebruik en dieselfde koeie is ook gebruik as rumenvloeistofskenkers vir die *in vitro* proewe.

In die eerste proef is die *in sacco*-prosedure gebruik om die kinetiese degradeerbaarheids-parameters oor tyd te bepaal. Al drie OKM substrate is gebruik en rumeninkubasies is in vier koeie gedoen. Inkubasietye was 0, 2, 4, 8, 16, 24 en 48 ure en die 0-ure waardes is verkry deur die onderskeie substrate in dacronsakkies in water te was. Droëmateriaal- en RP degradeerbaarheidsdata is met behulp van ‘n nie-lineêre model ontleed om die kinetiese parameters en effektiewe degradeerbaarhede te bepaal. Die proteïenbindmiddel het nie weerstand teen mikrobiese degradeerbaarheid verhoog nie. Die 0-ure waardes het wel verhoog ná Bioprotect® behandeling en dit het hoër model-afgeleide a-waardes tot gevolg gehad. In vergelyking met die kontrolebehandeling, is die effektiewe RP-degradeerbaarheid ook in al die substrate deur Bioprotect® behandeling verhoog.

In die tweede proef is ‘n Daisy^{II} incubator (ANKOM Technology, New York) vir die *in vitro*-inkubasies gebruik. Dieselfde drie OKM substrate is gebruik en inkubasietye was ook dieselfde. Behandeling * tyd interaksies het daarop gedui dat die proteïenbinder die RP

degradeerbaarheid van kanolameel na 8 ure inkubasie verlaag het (49.3 teenoor 54.2% vir die Kontrole; *P* = 0.022), asook na 16 ure (63.3 teenoor 67.5% vir die Kontrole; P = 0.04). In die geval van soja OKM het die behandeling geneig (P = 0.06) om die RP degradeerbaarheid na 16 ure inkubasie te verlaag (72.6 vs. 77.9% vir die Kontrole). Die afleiding is gemaak dat Bioprotect® die RP oplosbaarheid verhoog, maar die invloed van behandeling op RP degradeerbarheid is onduidelik a.g.v. verskillende neigings wat in die *in sacco*- en *in vitro*-proewe waargeneem is. Die verskil in die ordegrootte van RP degradeerbaarheid wat vanaf 4 tot 16 ure inkubasie tussen behandelings waargeneem is, dui wel daarop dat Bioprotect® ‘n onderdrukkende invloed op die degradeerbaarheid van die potensieel degradeerbare RP fraksie mag hê, maar dat hierdie invloed moontlik oorskadu word deur ‘n toename in die oplosbare RP fraksie wat met die Bioprotect® behandeling waargeneem is.

Die derde proef is gedoen om die invloed van behandeling op intestinale proteïen-verteerbaarheid te bepaal deur gebruik te maak van die Ross-analisemetode. Al drie proteïensubstrate is weereens gebruik en vir die ruminale inkubasiefase is rumenvloeistof van ses lakterende Holsteinkoeie verkry. Die eerste fase van die analise verteenwoordig ‘n 12-ure rumen-inkubasie, gevolg deur ‘n een-uur gastriese verteringfase en uiteindelik ‘n 24-ure intestinale verteringfase. Resultate het daarop gedui dat behandeling geen invloed op die totale intestinale proteïenvertering gehad het nie.

Die vierde proef is gedoen om die invloed van Bioprotect®-behandeling op die RP oplosbaarheid van die onderskeie substrate te ondersoek. Elke OKM is geïnkubeer in ‘n boraat-fosfaatbuffer vir een uur. Monsters is daarna slegs ontleed vir N-inhoud. Bioprotect® behandeling het proteïenoplosbaarheid en die oplosbare proteïeninhoud van soja OKM verhoog, maar het geen invloed op die ander oliekoeke gehad nie. Die oplosbare proteïeninhoud van soja OKM was 20.2% vir die Bioprotect® behandeling teenoor 13.1% vir die Kontrole (*P* < 0.001).

Die vyfde, en finale proef, is gedoen om die degradeerbaarheid van oplosbare proteïen te bepaal. Slegs soja OKM is in hierdie proef gebruik, aangesien dit die enigste substraat is waar die oplosbare proteïeninhoud deur behandeling beïnvloed is. Dieselfde prosedure is gevolg as dié in Proef 4 om oplosbare proteïen vir die hierdie proef te verkry. Die oplosbare proteïen supernatant is daarna by ‘n gebufferde rumenvloeistof-inkubasiemedium, soortgelyk aan dié van die *in vitro* proef hierbo beskryf, gevoeg en die monsters is vir 0, 2, 8 en 24 ure by 39°C geïnkubeer, gevolg deur N-analises. Bioprotect® het die degradeerbaarheidstempo, sowel as die hoeveelheid RP wat gedegradeer is, betekenisvol verlaag. Tydens die totale 0 tot 24-ure inkubasieperiode, was die degradeerbaarheidstempo

(k_d) 0.028/h^{-1} vir die Bioprotect®-behandeling en 0.036/h^{-1} vir die kontrolebehandeling, wat duidelik op die neerdrukkende effek van Bioprotect® op die degradeerbaarheid van die oplosbare proteïenfraksie dui.

Die finale gevolgtrekking is gemaak dat die proteïenbinder, Bioprotect®, die potensiaal het om ruminale RP-degradeerbaarheid in sekere oliekoeke te verlaag. In die geval van soja OKM, wat een van die belangrikste proteïenbronne in melkbeesdiëte is, het Bioprotect®-behandeling die degradeerbaarheid van die oplosbare proteïenfraksie aansienlik verlaag. Verdere navorsing met lakterende melkkoeie is geregverdig om die invloed van Bioprotect® op melkproduksierespons te ondersoek, veral indien die RP-inhoud van die dieët verlaag word.

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a	Soluble and rapidly degradable fraction
AA	Amino Acids
ADIN	Acid detergent insoluble nitrogen
b	Potentially degradable fraction
c	Rate of degradation of fraction b
CM	Canola meal
CSND	Corn silage neutral detergent
CP	Crude Protein
DDG	Dried distillers grain
DDGS	Dried distillers grain and solubles
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EAA	Essential amino acid
eDegCP	Effective crude protein degradability
effDeg	Effective degradability
GIT	Gasto-intestinal tract
IADP	Intestinally absorbable dietary protein
ID	Intestinal digestibility
k_p	Passage rate from the rumen
k_d	Digestion rate
OCM	Oilcake meal
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
RPAA	Rumen protected amino acids
MBT	Mobile bag technique
MP	Metabolisable protein
MNB	Mobile nylon bag

MTS	Modified three-step
MY	Milk yield
N	Nitrogen
ND	Neutral detergent
NDF	Neutral detergent fiber
OTS	Original three step *in vitro*
VFA	Volatile fatty acids
NPN	Non-protein Nitrogen
FCR	Feed conversion ratio
SBM	Soybean oilcake meal
SFM	Sunflower oilcake meal
SEM	Standard error
TMR	Total mixed ration
TP	True protein
RUP	Rumen undegradable protein

Chapter 1

General introduction

Proteins are made up of 50 or more amino acid (AA) chains. Cows have a certain requirement for each individual AA to execute different functions including maintenance, growth, reproduction and milk production (De Ondarza *et al*., 2004). There is a great interest in the balancing of dairy cattle diets for AA chains. Increases in N intake, milk N and milk protein may be potentially beneficial as it may contribute to farm profitability while reducing environmental impacts. The AA's available for absorption in the small intestine are supplied by microbial protein, ruminal undegradable protein (RUP) and endogenous protein in sufficient quantities to high producing dairy cows in order to meet metabolisable protein requirements (Paz *et al*., 2014).

The protein component in the diet of dairy cows is widely accepted as the most expensive nutrient. Dietary protein plays an important role in supplying AA's and N for microbial protein synthesis. The total feed cost is dependent on the balance of requirement and supply of protein, as well as the chosen protein source in the diet (Imran *et al*., 2018). Bypass protein levels vary in different feedstuffs and the cost of bypass protein could amount to about 30 percent more than merely total crude protein (CP). Fish meal, for example, has a CP content of more than 600 g/kg, of which about 65% escapes rumen degradation (Guthrie and West, 1991). Despite the high nutritional protein quality, the high cost of fish meal limits its inclusion in ruminant diets. The high cost of bypass protein sources has resulted in an ongoing search for alternatives (Wright and Lackey, 2003).

Rumen degradable (RDP) and undegradable (RUP) protein define dietary protein. Non-protein nitrogen (NPN) and some true protein contribute to the RDP fraction which is degraded by microorganisms in the rumen. Undegradable protein passes to the small intestine and is protected from ruminal degradation (Bach *et al*., 2005). Protein nutrition for dairy cows includes a requirement for both RDP and RUP. In diet formulation, attempts are made to optimise RDP:RUP ratios in order to lower the total CP content without compromising production efficiency. The inclusion of digestible RUP in diets has true value to the amount absorbed AA (NRC, 2001). The protein requirement of high yielding dairy cows can only be met when the required amount of RUP is supplied (De Ondarza, 2004) and microbial protein alone cannot meet the requirement for metabolisable protein in such cows (Hedqvist *et al*., 2006).

Protein that is protected against ruminal degradation flows to the small intestine where it is digested enzymatically and the AA's absorbed. Many studies have been reported in which protein protection was achieved through using heat and/or chemical treatment for a number of oilcake meals (NRC, 2001). Oilcake meals included in dairy diets aim to provide high quality protein. Soybean, sunflower, canola and cottonseed meals are the most abundant that are produced and used in the South African animal feed industry (DAFF, 2017). Heat treatment of protein sources, however, may result in a loss of lysine and cysteine, because of their heat sensitivity (Ljøkjel *et al*., 2000).

Many feedstuffs have a low RUP content; therefore, the provision of a protein supplement or protected protein is of importance in dairy cow nutrition (NRC, 2001). Protein feeds differ in their degree of ruminal degradation. Degradation rates have been determined to ensure optimal formulation of dairy cow diets (Mohamed *et al*., 2008). *In sacco, in vitro* and *in vivo* techniques have been used to determine a reasonable estimate for protein degradability in the rumen (Van der Walt *et al*., 1988). According to Mohamed *et al* (2008), the *in sacco* technique is the most effective, while *in vitro* has been shown to be a good alternative. The *in vitro* method is less expensive but is still dependent on cannulated animals for the use of rumen fluid.

Formulating dairy cow diets to meet protein requirements has shifted from formulating for CP alone to that of metabolisable protein (MP), which is digested and absorbed as AA in the small intestine (Ross *et al*., 2013). The MP is derived from RUP, microbial protein and endogenous protein (De Ondarza, 2004). Improving accuracy of diet formulation would result in more space for other raw materials and would be more economical (JHC van Zyl, 2019; personal communication). Determining accurate intestinal protein digestibility estimates is thus important to predict and ensure an adequate intestinal AA supply. A few *in vitro* methods are available to determine intestinal digestibility, but a re-development of these methods was done by Ross *et al* (2013) for protein feedstuffs used in dairy nutrition.

Soluble protein is the amount of CP of a feed being dissolved when entering the rumen. This amount of CP is usually accepted to be rapidly digested by rumen microbes. However, according to Hedqvist *et al.* (2006), proteins differ in the rate of soluble protein degradation. The soluble protein part of a feed can be determined by mixing a phosphate-borate buffer solution with a sample of the feedstuff (Western Dairy Science, 2004). Degradation of the soluble protein cannot, however, be determined by the *in sacco* method and alternative ways have to be explored.

The objectives of the current study were therefor to determine the effect of a potential protein binder on:

- *in sacco* and *in vitro* protein disappearance parameters
- protein solubility and the degradability of soluble protein and
- intestinal protein digestibility

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Chapter 2

Literature review

2.1 Introduction

Protein plays an important role in almost all biological processes, for example transport, storage, immunology, mechanical support and metabolism control (Van der Walt and Meyer, 1988). The stomach of the ruminant consists of four compartments, each with a different function. The rumen, which is the biggest of the four, is the habitat of the microbial population. Ruminal digestion is defined as a dynamic process of food entering ruminal fluid and resulting in an output of microorganisms, food not degraded and fluids (Andrade-Montemayor *et al*., 2009). Rumen microorganisms degrade plant protein from the diet to various degrees and synthesise protein from the resulting ammonia, namely microbial protein. Microbial protein and protein not degraded in the rumen pass to the small intestine, where they are broken down to amino acids (AA) and where absorption takes place (Rounds and Herd, 1987). The protein requirement for ruminants is primarily supplied by microbial protein (Wattiaux, 1994).

Supplementing protein is an expensive cost added to the diet. The use of protein in the ruminant system can be optimized to increase the production of the animal or decrease the quantity of protein inclusion in the diet (Tandon *et al*., 2008). By decreasing the protein quantity in the diet, more space for other raw materials would be available (JHC van Zyl, 2019; personal communication).

Dietary protein not degraded in the rumen, commonly referred to as bypass protein, is utilized in the small intestine to contribute towards the metabolisable protein requirements of the animal. Rumen undegradable protein (RUP) is required by the dairy cow to meet the requirement for absorbed protein. The requirements of protein for high milk production cannot be supplied by microbial protein alone and is supplemented by RUP to meet requirements (De Ondarza, 2004). Different feedstuff has different natural bypass protein values. Fishmeal, cottonseed cake, coconut meal and maize gluten meal are some of the feeds with high bypass protein value (Walli *et al*., 1995).

The balance between the RDP and RUP is important for the efficient utilisation of nitrogen. An excessive amount of RDP or RUP would result in wastage of nitrogen and be excreted

into the environment. The protein not absorbed is excreted in the urine. Only 45% of the protein from RDP is absorbed in the small intestine as AA and it has an absorbing percentage of about 85% in the small intestine as AA (Stallings, 2002).

There are a number of ways to protect protein against ruminal degradation. Heat treatment or using a chemical reagent are most commonly used methods (Walli *et al*., 1995). There are a few methods that can be used to determine the degradability of feed proteins (Mohamed and Chaudhry, 2008), each one with its own advantages and disadvantages. The nylon bag technique is the most widely used technique to determine the degradation of protein (Walli *et al*., 1995).

2.2 Protein digestion and metabolism

Crude protein (CP) is defined as the dietary protein in a feedstuff and it consists of true protein and non-protein nitrogen (NPN). Dietary protein is the CP ingested via the feed. In a ruminant animal, dietary CP may be degraded in the rumen by microorganisms at a certain rate, depending on factors such as feed degradability and passage rate from the rumen (Bach *et al*., 2005). The degradable fraction is hydrolysed in the rumen and available for microbial protein synthesis, also referred to as microbial protein (Andrade-Montemayor *et al*., 2009). The feed CP fraction resistant to ruminal degradation passes to the small intestine to contribute towards the metabolisable protein (MP) requirement (Wattiaux, 1994).

2.2.1 Rumen degradable protein (RDP)

The end products of protein degradation in the rumen by microorganisms include branched chain fatty acids, peptides, AA and ammonia, which are major sources of nitrogen for microbial growth (Wattiaux, 1994). The microbes use these N sources, as well as energy from carbohydrate digestion, to form their own protein, called microbial protein, which is used for growth, production and reproduction (Wattiaux, 1994). The bacteria in the rumen attach to the feed particles and undergo proteolysis. Smaller peptides and AA are transported inside microbial cells. The AA can be incorporated into microbial protein or deaminated to volatile fatty acids (VFA), CO_2 and ammonia. Peptides can be degraded to AA through peptidases. The AA can either be used directly for microbial protein synthesis or trans-aminated, if energy is available. When energy is limited, AA will be deaminated and their carbon skeletons will be fermented into VFA. Peptidolysis and deamination play an important role in controlling degradation (Bach *et al*., 2005). The rumen microbes are then flushed from the rumen, through the reticulum and omasum, to the abomasum where they are killed by the acidic environment. The amount of microbial protein that is flushed to the abomasum depends mainly on the amount of N and energy available in the diet (Moran,

2005). The resulting protein supplied by microbial and undegraded protein, collectively referred to as metabolisable protein, is digested to AA and absorbed in the small intestine to be used by the animal for growth, reproduction, milk production and maintenance (Das *et al*., 2014). Some VFA's are produced from fermented AA when the carbohydrate requirements are not met (De Ondarza, 2004).

Highly degradable protein or an excessive amount of protein in the diet leads to an excess amount of ammonia in the rumen, some of which will be transported to the liver and converted to urea, which is released into the blood. This process occurs when an excessive amount of ammonia is produced, more than what the rumen microbes can use. Two routes can be followed for urea to return back to the rumen, through saliva or the rumen wall (Parish *et al*., 2009). Urea that reaches the rumen again is converted to ammonia and can be used for bacterial growth. Too much protein in a diet will lead to less recycling of urea and more excretion in urine by the kidneys. A deficit of protein in the diet will cause more urea to recycle to the rumen, where it is available for microbes to produce microbial protein (Wattiaux, 1994). The recycling process is a waste of protein and energy, because conversion of ammonia to urea in the liver comes at an ATP cost (De Ondarza, 2004).

Bacteria and protozoa are found in the rumen, which produce proteolytic enzymes that degrade protein. Proteolytic activity is normally linked with bacterial cells. The rate of proteolysis and the retention time in the rumen determine the rate of protein degradation. The proteolysis is more important than the retention time, but the protein exposure time to enzymatic activity plays an important role in the degradability (Van der Walt and Meyer, 1988).

Solubility of rumen degradable protein:

The classification of soluble or insoluble protein is an important factor, because more nitrogen is available for rumen microorganisms to use from soluble proteins (Andrade-Montemayor *et al*., 2009). Solubility is a very important factor for protein sources and gives a good indication of their functionality (Zayas, 1997). Small AA chains or NPN solubilize in the rumen fluid and is absorbed across the rumen wall (Heeg, 2016). Proteolysis, the protein breakdown to AA or smaller peptides, occurs more with soluble proteins than insoluble protein.

Four types of protein are found in protein supplements, namely albumin, globulin, prolamins and glutelins. Albumins and globulins have a higher biological value having a better AA

composition. Albumin and globulin are soluble in rumen fluid while prolamins and glutelins in contrast are less soluble. High molecular weight, disulphide bonds and more cross-linking are found in prolamins and glutelins, which make them less soluble (Clark *et al*., 1987). Disulphide bonds are found with some soluble albumins, which cause a slow degradation in the rumen (Bach *et al*., 2005).

Protein solubility and degradability is not the same (Stern *et al*., 1994). Previous *in vivo* studies done by Stern and Satter (1984) have shown that there is a correlation of about 0.26 between solubility of N and protein degradability. The soluble fraction of feedstuffs has different degradation rates. Approximately 50% of the soluble fraction in some feedstuffs is resistant to degradation in the rumen and will be digested in the small intestine (Dyck *et al*., 2015).

Table 2.1 shows that the protein degradation in feedstuff differs, as well as the difference between soluble protein and degradability.

Table 2.1 Calculated RDP and RUP (Dyck et al., 2015).

% of total protein	Canola meal	Linseed meal	Field seed peas	Soybean meal
Soluble protein	24.4	28.6	77.8	16.9
RUP	44	45	71	68
RDP	56	55	29	32

2.2.2 Undegradable dietary protein (RUP)

The protein that escapes ruminal degradation and protein that is directly available to the cow is known as RUP (Moran, 2005). Cows have a requirement for absorbed protein which can be met when the total protein is enough, by supplementing microbial protein with an amount of RUP. Heat or chemical treatments to the protein are common procedures to increase the amount of protein that would be resistant to degradation (De Ondarza, 2004). Protein digestion starts with acid-pepsin digestion in the abomasum, where protein is broken down to AA and absorbed by the small intestine with pancreatic and intestinal proteases (Stern et al., 2006). This results in a contribution to the metabolic protein pool, which is available to the animal for use. The amount of different AA provided by RUP is more than that of microbial protein (Moran, 2005). Each protein source's level of rumen degradation differs, where by-products normally consist of a higher resistance to rumen degradation than forages. At least 40% of the AA absorbed by the small intestines is from RUP (Wattiaux, 1994).

2.2.3 Metabolisable protein (MP)

Protein that flows from the rumen and is digested and absorbed in the small intestine as AA is known as metabolisable protein (MP). Microbial protein, RUP and endogenous protein all contribute to the MP. Microbial protein contributes almost 60% to the MP while the other 40% is supplied by the RUP (De Ondarza, 2004). All excess AA are transported to and broken down in the liver. In the case of lactating cows a portion moves to the mammary gland to produce milk protein. The AA is used for muscle growth in young growing animals and used for the calf in pregnant cows. Tissue growth and tissue turnover (the process where tissues are replaced by new tissues) are also a major function (WDSI, 2004).

2.2.4 Non-protein nitrogen (NPN)

Non-protein nitrogen is not a true protein and does not contain AA but nitrogen that can be used by ruminants. Non-protein nitrogen is converted to ammonia in the rumen and used by the rumen microbes to form microbial protein. Some of the microbes have a specific requirement for ammonia. The microbes use carbohydrates when they are available to incorporate AA and NPN and produce microbial protein (De Ondarza, 2004).

Urea and anhydrous ammonia are examples of NPN. Urea, which contains about 47% nitrogen, is rapidly hydrolysed to ammonia by urease from ureolytic bacteria. The resulting ammonia can be used to synthesise a significant amount of microbial protein (Cassel, 1996) while surplus ammonia is absorbed via the rumen wall and transported to the liver. Amino Acids resulting from protein degradation may also combine with ammonia and carbohydrate metabolism products to form microbial protein (Stanton and Whittier, 2006).

Non-protein nitrogen is not as expensive as plant or animal based proteins. Non-protein nitrogen is added to the grain mix with forages, but makes up a small percentage because of low palatability (Cassel, 1996). About 50% of the total N in maize silage can be contributed from NPN and 10 to 20% NPN in the case of alfalfa hay. The inclusion of more nitrogen than needed in the diet cannot be used or stored and must leave the body. When more N is consumed than required, the excretion systems can be damaged, overloading of AA deamination and the capacity of detoxifying the liver occurs. The inclusion of N at a lower level than needed to meet the requirements, will cause a decrease in feed conversion ratio (FCR) and growth rate (Karcol *et al*., 2016).

Urea is one of the products of protein metabolism in mammals (Figure 2.1). Some urea produced by the animal is excreted through urine/milk or returns to the digestive tract via saliva. Non-protein nitrogen occurs in components, such as DNA, RNA, AA, ammonia and small peptides but only nitrogen from ammonia, AA and small peptides are used for microbial growth (Bach *et al*., 2005).

Urea poisoning may occur when high levels are included in diets and it can result in deaths. Symptoms include rapid breathing, bloat, tetany and an increase in rumen pH (8) and blood ammonia levels (Stanton and Whittier, 2006).

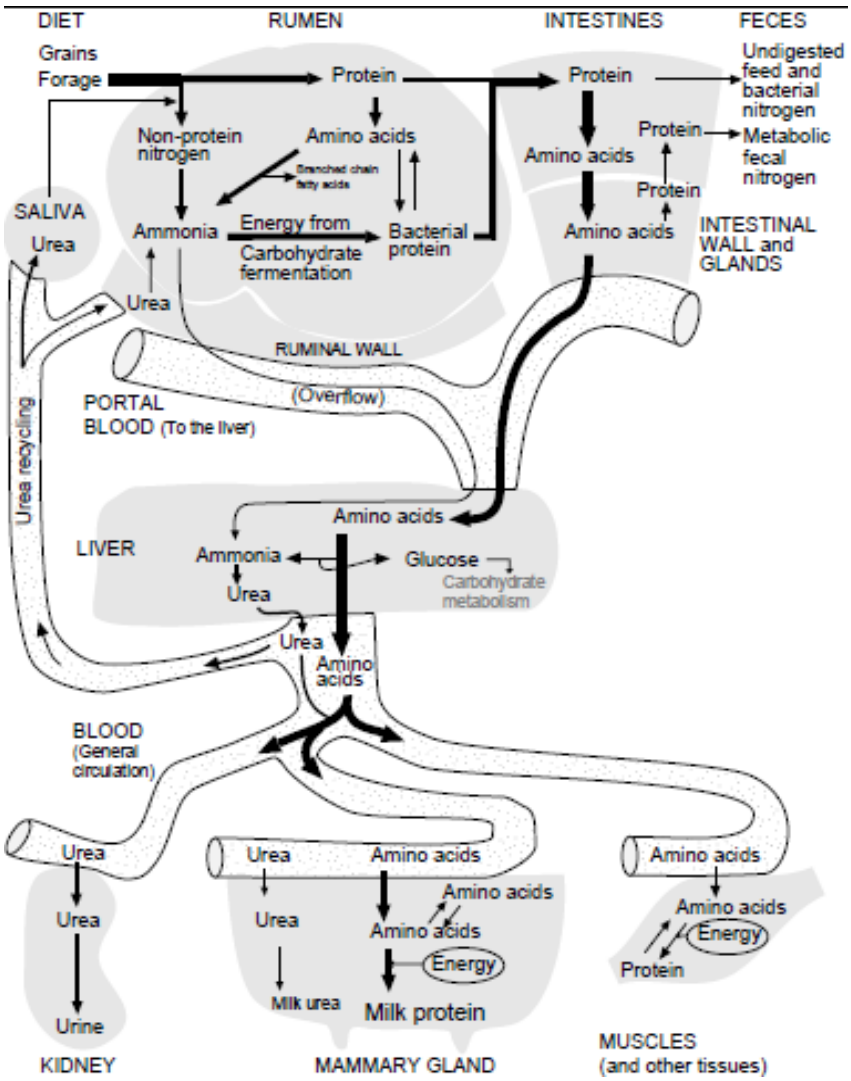

Figure 2.1 Protein metabolism in the dairy cow (Wattiaux, 1994).

2.3 Determining rumen undegradable protein (RUP)

The degradability in the rumen and intestine of ruminants are required by the protein evaluation systems (Paengkoum *et al.,* 2013). In ruminants the dietary proteins are degraded in the rumen by microbial fermentation or in the small intestine by enzymatic hydrolysation. The feedstuff and animal characteristics determine the extent of degradation. There is no totally accurate procedure to determine the rumen degradability of feedstuffs for ruminants (Van Straalen *et al*., 1993). The disappearance of DM or N from feedstuff in nylon bags situated in the rumen of cannulated animals can be measured. The measurement of the amount of nutrients flowing to the small intestine can also be used to determine the degradability. There is interest in an alternative approach to the *in sacco* method, which is both less expensive and more convenient, namely the *in vitro* (Mohamed and Chaudhry, 2008). Both approaches will be discussed.

2.3.1 *In sacco*

A certain amount of dietary protein escapes rumen degradation. One of the most widely used techniques to determine the extent of feedstuff degradation in the rumen is by using the *in sacco* nylon bag technique, also known as the *in situ* method. Small amounts of a specific feedstuff are incubated in the bags in the rumen of cannulated cows for incremental periods of time. The correct procedure and equipment need to be followed to have reliable data. The combined information of disappearance rate of nitrogen and the out-flow rate to the small intestines from the rumen, is obtained from this technique (Ganev *et al*, 1979). There are some factors influencing the results, such as the sample weight, sample processing, size of the bags, size of the pores, washing method, microbial N contamination of bags and the diet of the host animal.

The fibre bag technique was used by Quin *et al*. in 1938 for the first time and it was done on cannulated sheep. The bags were cylindrical and natural silk. The use of artificial fibre bags was first used by Erwin and Elliston in 1959 and improved the understanding of the degradation of feed components in the rumen (Ørskov *et al*, 1980).

Not only is the simplicity of this method an advantage, but the number of samples that can be handled simultaneously, makes it a popular technique (Pienaar *et al*., 1989). Another advantage is that the digestive system of a live animal is used, so there is less chance of error in comparison to other techniques (Stern *et al*, 1994). The rate of degradation in the rumen is also measured by this technique (Ørskov *et al*, 1980). According to Ørskov and Shand (1997), the technique is also used for roughage evaluation, which identifies the factors of plants (soluble, insoluble and fermentable fractions) affecting the roughage

consumption. Optimal NH_3 and S concentrations, as well as the optimal pH can also be determined with this method. The requirement of measurements is fewer with *in sacco* studies in comparison with other techniques.

The *in sacco* approach is, however, not without problems. For example, the samples in the bags are not exposed to chewing and rumination, which means that no mechanical breakdown will occur as would have been the case if it were fed to the animal. The samples will also not be able to leave the rumen when a certain particle size is reached, as it would in normal situations. The actual measurement is the breakdown of particles to a very small size to be able to leave the bag (Ørskov *et al*., 1980). Not only is the microbial contamination an error that affects the DM and N disappearance, but the size of the sample in the bag as well. Studies have shown that an increase in sample weight reduced DM digestibility with short incubation times (Mohamed and Chaudhry, 2008).

The nominal pore size of the bags is not fixed and can range from 10 µm to 53 µm (Ørskov *et al*., 1980). Pore size smaller than 15 µm causes decreases in degradability because of the restriction of microbial colonisation and diversity as well as trapping of fermentation gases. Pore sizes bigger than 40 µm can cause a loss of undegradable particles and solubles. The size of the sample to bags ratio should be correct to ensure that no overfilling or under filling occurs. This technique is useful if the bag is large enough to allow for free movement of the substrate (Mohamed and Chaudhry, 2008).

2.3.2 *In vitro*

Alternative methods for *in sacco* or *in situ* trials include different types of *in vitro* approaches which may involve buffers, chemical solutions, rumen fluid and enzymes. Such methods can be used to estimate rumen degradation and fermentation parameters, either directly or via gas production trials. The methods may, however, not meet realistic expectations (Taysom, 2013), although a high degree of correlation is usually found between *in vitro* and *in vivo* trials for DM digestibility (Holden, 1999).

The advantage of *in vitro* trials is that a number of samples can be done simultaneously, which means that the amount of labour is reduced while increasing the precision of this technique (Holden, 1999). *In vitro* studies are not expensive and they provide the opportunity to analyse the residue and the metabolites of microbial degradation. Factors influencing degradation, such as the environment, microbial and animal factors are more controllable than with other methods (Mohamed and Chaudhry, 2008).

The buffer solution, macromineral, micromineral, incubation medium and reducing solution are usually prepared according to Goering and Van Soest (1970). *In vitro* incubation can be

done in fermentation tubes, Erlenmeyer flasks or in a DAISY II incubator used for *in vitro* procedures and is done according to ANKOM protocol. The rumen fluid to *in vitro* solution ratio is 1:4 and samples are incubated at 39.5˚C for specific incubation times (Holden, 1999). Rumen fluid is collected from ruminally cannulated animals (Mohamed and Chaudhry, 2008).

2.3.3 *In vivo*

In vivo refer to live animals and the method is currently not as popular as the *in sacco* or *in vitro* methods. However *in vivo* trials are trustworthy and preferred in terms of protein degradability and feeding value (Acar, 2018). The costs and risks related to *in vivo* studies are high and they are more labour intensive than *in sacco* and *in vitro* experiments (Stern *et al*., 2006). Furthermore, the large quantities of feed required and the number of replications play an important role in the total costs. Multiple animals are normally used for *in vivo* studies and the maintenance cost is expensive. These expenses make this technique impractical (Filho *et al*., 2003).

2.4 Determination of intestinal digestibility

Dynamic models estimating nutrient balance and supply, as well as requirements, are becoming more accurate and precise. Diet formulation for dairy cows to meet protein requirements are firstly done according to CP requirements before addressing the contribution of metabolisable protein and AA availability. Estimating the intestinal digestibility (ID) of protein and AA accurately is becoming more important to supply nutrients for optimum production. Outdated data used for MP requirement may lead to over or under supply. Previous published *in vitro* methods were modified by Ross *et al.* (2013) to develop a laboratory system for the estimation of ID. The procedure has become known as the Ross assay.

In vitro digestion methods started with a two-step procedure by Tilley and Terry (1963) who combined ruminal digestion and pepsin digestion. A three-step *in situ* bag technique was developed by Calsamiglia and Stern (1995) where the bag residues were exposed to HCl and pepsin digestion, before introducing a pancreatin digestion step. This procedure was modified by Gargallo (2006) who used the ANKOM Daisy incubator.

The control substrates used in the Ross assay are CSND (corn silage neutral detergent), freeze dried blood (positive control) and burnt blood meal (negative control). The CSND is used as a fermentation control sample, which accounts for microbial contamination. The burnt blood meal is used as the negative control, because of their low (almost negligible)

ruminal and intestinal digestibility whereas the freeze-dried blood serves as a control to verify maximum N digestibility.

2.5 Methods to increase bypass or RUP values of protein feedstuffs

Undegradable dietary protein (RUP) is one of the two metabolizable protein sources for ruminants, the other one being bacterial protein. Feedstuffs that are treated to increase RUP values contain high levels of CP. Bypass techniques are used to improve the effective utilization of protein. Nitrogen and energy balance are increased with protected proteins, which leads to a higher milk yield. The contribution towards the total metabolizable protein supply is much higher when a bypass technique is used. About 80% of the protein that passes from the rumen is digestible in the small intestine (Harmon *et al*., 1986).

There are different treatments that can be applied to protect protein from degradability in the rumen. These include physical treatment, chemical treatment and more recently, protein binders. To choose one of these treatments, one would have to look at the cost and how effective the product is protected, based on scientific studies. The success of bypass treatment would be apparent when less protein is included in the diet without compromising milk production, thus saving costs (Harmon *et al*., 1986).

2.5.1 Physical treatment

2.5.1.1 Heat treatment

The drying of forages with heat is known to prevent some of the protein to be degraded in the rumen. The heat that applied during the manufacturing of oilseed meals varies in temperature (°C) which determines the different degrees of protection. Effective protection against degradability in the rumen is provided when denaturation of the protein occurs. Denaturation happens when intensive heat is applied to protein. By providing heat to the protein supplement for 2 to 4 hours at 125 to 150˚C would result in a high degree of protection (Tandon *et al*., 2008). The Maillard reaction occurs between sugar aldehydes and AA's, and acid detergent insoluble nitrogen (ADIN) is increased with extended heating time. The peptide linkage between AA's and the two α-amino acids, glutamine and asparagine, are more resistant to enzymatic hydrolysis when heated. High temperature heating of proteins may cause a loss of lysine, arginine, cysteine, methionine and tyrosine. The lysine is made indigestible by the reactions between the amide groups of glutamine and asparagine and the ε-amino group of lysine (Kamalak *et al*., 2005). Heat treatment is cost effective and one of the most practical methods (Mudgal and Sengar, 1984). Trials that were

done with sheep and calves showed that the rumen ammonia concentration levels were low, and an improvement of nitrogen balance or better growth were observed. In dairy cows, this has also had a positive effect on milk yield. The flow of protein to the small intestine may be increased by heat treatment, but too much heat may result in a lower quantity of some AA's and may also decrease the digestibility in the small intestine (Kamalak *et al*., 2005).

2.5.1.2 Encapsulation

Encapsulation can be applied on a variety of solids, gases or liquids with a very small capsule. Nutrients, enzymes, drugs, feedstuff or bacteria can be encapsulated. Because of the additional costs involved, protein encapsulation is only done on protein sources that have a high biological value (Tandon *et al*., 2008). The coating can vary in thickness, depending on where in the gastrointestinal tract (GIT) the release should take place. A barrier is created between the encapsulated substance and the (rumen) environment. The polymer is soluble in the more acidic conditions such as the abomasum but will be insoluble in the rumen. Protection against rumen microbial degradation is not the only advantage of encapsulation, but it could prevent oxidation and enzymic degradation of nutrients as well. The masking of odours and taste is another advantage of encapsulation that is often applied in the food industry (Emanuele *et al*., 2006).

Encapsulating AA has become quite common. In a study by Rogers *et al.* (1987), encapsulation was done for the individual AA's methionine and lysine. Lysine that bypassed the rumen increased the feed intake, milk yield and the utilization of methionine. The production of milk protein was increased by both of these rumen protected amino acids (Rogers *et al*., 1987). Encapsulation of methionine was done by Papas *et al.* (1984) and it was shown to protect the AA successfully and to deliver it post ruminally for enzymatic digestion.

2.5.2 Chemical treatment

2.5.2.1 Formaldehyde treatment

This is the most widely used chemical treatment for protein protection. High quality protein treated with formaldehyde forms cross-links with the amino groups and results in a less susceptible protein to microbial attack. Such protein has been shown to be more digestible in the small intestine. The solubility of protein is reduced at pH 6 and much will pass to the small intestines for digestion, where the bonds are broken under the more acidic conditions (Tandon *et al*., 2008). The decrease in urinary nitrogen and increase in faecal nitrogen excretion indicate that the protein protection is effective. This treatment might be beneficial

for the animal, but in certain cases, especially when over processed, it could decrease productivity, because of a reduction in the synthesis of microbial protein. Over processing can thus have a negative effect on the nutritional value and a decrease in protein digestibility in the small intestine (Kamalak *et al*., 2005). Other aldehydes that can be used are not as easily available and more expensive than formaldehyde (Tandon *et al*., 2008).

2.5.2.2 Tannic acid

Secondary compounds that have anti-nutritional properties, such as tannins, are found in many plants. The reason for tannin to be anti-nutritional is because of the negative effect on protein digestibility (Wina and Abdurohman, 2005). Furthermore, tannins may result in lower feed intake, rate of protein and fibre breakdown and efficiency of microbial protein synthesis (Nsahlai, 2011). Tannins can be divided into two groups, namely hydrolysable and condensed tannins. On the positive side, the binding of tannin to protein may result in an increased protection of protein against rumen degradation, which could lead to increased small intestine digestion and thus increased productivity (Wina and Abdurohman, 2005; Yusiati *et al*., 2018).

The types of binding between tannins and proteins can be hydrogen bonds, covalent bonds, ionic bonds or hydrophobic interactions. The binding between tannin and protein is a complex process involving multiple sites for protein to bind, because of the high degree of hydroxylation. The stability of the binding is pH dependent and at a low pH the complexes are capable to release protein (Hagerman and Butler, 1981). Tannins usually bind to protein but can also bind to starch and structural polysaccharides. These bindings may result in lower nutrient bioavailability in the digestive tract and seen as anti-nutritional, because of the decrease of protein digestibility. The flow of protein to the small intestine and dissociation in abomasum occurs because of the pH dependent, reversible tannins and protein binding (Martínez, 2005).

Ethiopian Menz sheep used in one study received oilseed cake meal, followed by tannin rich browses. The sheep gained more weight than those being fed the diet in the reversed order. The protein and tannin interactions and the proteolytic activity being depressed were the reason for more protein bypassing the rumen (Nsahlai, 2011).

2.5.2.3 Protein binders

A product that has shown to provide protection against ruminal degradation of highly digestible starch components of feed is BioProtect® (Realistic Agri). BioProtect® is a dark liquid and is a stable non-volatile organic salt, which reacts with primary or secondary amino

groups of protein and hydroxyl groups of starches. The aldehyde (HCHO) reacts with the amino groups, but H_2SO_3 being produced by hydrolysis of Bioprotect®, can react with amino groups as well. At a neutral pH the complexes are stable, typically in the rumen where the pH ranges between 6 and 7. In the abomasum and small intestine, where the pH drops to 2 or 3, the bonds dissociate for effective digestion and absorption of nutrients (Dunshea *et al*., 2012).

According to the suppliers (Realistic Agri), the protein protection ability of Bioprotect® in the rumen is much stronger than that of formaldehyde treatment. They also claim that the active ingredient is three times more reactive than formaldehyde, because of the three double-bond oxygen atoms.

Dunshea *et al* (2012), Gonzalez *et al* (2014) and Van Zyl (2017) have done studies on starch treated with the protein binder. The undegraded starch passed to the small intestine successfully.

2.6 Protein sources

2.6.1 Plant protein by-products

2.6.1.1 Oilcake meals

Canola oilcake meal

This high-quality protein feed is used by dairy farmers as well as beef cattle farmers. The high protein quality makes it a popular feed to use for milk production and growth (CCC, 2015). This feed is a by-product and is produced after oil has been extracted through physical or solvent extraction (Mailer, 2004). Its AA profile well balanced and contains high levels of histidine, methionine, cysteine and threonine (CCC, 2009).

Canola oilcake meal can be produced by different methods. Cold press is the separation of oil and the meal by physical actions without heat. Expelling is the physical extraction of oil and meal with heat. Both these methods result in an 8 to 12% oil residue and they differ in characteristics because of different temperatures (less than 60˚C) applied. The method which results in less than 1% oil residue is solvent extraction where physical expeller extraction precedes solvent washing. This method includes processes such as flaking, physical and solvent extraction, toasting, additives and heat treatment (Mailer, 2004). Canola expeller meal showed similar or better results for milk production than solvent extracted

canola meal (CCC, 2015). Crude protein and moisture content of the meal are the factors that play a role in the quality (Mailer, 2004).

Less than half (48.1%) of the protein in canola meal is degraded in the rumen (CCC, 2015).

Canola oilcake meal has a high palatability, which might be a result of the sucrose content, and it results in a higher DM intake than diets including other oilcake meals.

Cottonseed oilcake meal

Cottonseed oilcake meal is a protein source often included in dairy cow diets and known to be economical (Bangani *et al*., 2000). The extraction procedures applied to produce cottonseed oilcake meal resulted in a high-quality protein source of about 40% CP (Meric *et al*., 2011). This meal contains higher levels of RUP than soybean meal, but it is lower in essential AA concentrations (e.g. lysine). Studies have shown that replacing soybean meal with cottonseed meal had no decreasing effect on animal performance (Imaizumi *et al*., 2015). Cottonseed products contain gossypol, a phenolic compound, that can be toxic to monogastric animals and young calves where the rumen is not fully developed. The rumen can, however, detoxify gossypol by binding with soluble proteins or absorbing gossypol at a slower rate as a result of dilution (Bangani *et al*., 2000).

Sunflower oilcake meal

Sunflower seed is one of the major crops for oil production. Sunflower seeds are partially dehulled and oil is extracted to form sunflower oilcake meal. Hydraulic pressure or solvent extraction is used to remove the oil (McDonald, 2011). Dehulling can be controlled before extraction of the oil. The more hulls removed, the more the fibre content will decrease with a resultant increase in density of the meal (Nell *et al.,* 1993). By removing the hulls the CP content is increased and a more digestible meal is produced (McDonald, 2011). The higher protein types have a CP content of 38 to 42%, where the lower protein types have a CP content of around 28%. Palatability is not always high, and the bypass value of the protein is not high either.

The high fibre content of sunflower meal reduces the inclusion levels to about 3 or 4% in monogastric animal diets (McDonald, 2011). The maximum inclusion level for adult cattle is 200 kg/ton in diets and inclusion is not recommended for calve diets. Low lysine levels occur and high methionine levels, but it is a useful protein source (McDonald, 2011).

Soybean oilcake meal

Soybean oilcake meal is one of the best protein sources available to farm animals (McDonald, 2011). The meal appears pale yellow or light brown with flakes of irregular shapes (Soybean trading, 2016). The oilcake meal is a commonly found in the diet of lactating dairy cows as a protein source (Imran *et al*., 2018). The CP content of soybean meal is around 44% to 49% and the protein digestibility about 88% (Banaszkiewicz, 2011). The RDP value is high, and it has a good AA profile as well. Furthermore, the cell wall digestibility of the hulls is high and often included in ruminant diets. As a major protein source in dairy cow diets, soybean meal shows an increase in milk yield, milk protein content and dry matter intake (Imran *et al*., 2018).

Mechanical or solvent extraction methods are used for the production of soybean oil and soybean flakes. The soybean flakes are toasted or boiled to produce the final meal. The hulls are added back afterwards, which results in differences in the fibre and protein content of soybean meals (Banaszkiewicz, 2011). The production location, soybean variety and the process methods also cause soybean meals to vary in quality. The meals that do not contain hulls have a higher proportion of CP, AA and MP (Dozier *et al*., 2012).

Soybean meal contains all the essential AAs, with methionine being the first limiting AA. Soybean meal has high levels of arginine, leucine and lysine (Banaszkiewicz, 2011). Despite having a deficiency in B vitamins, it is still a valuable protein source for animals and widely used (McDonald, 2011).

2.6.1.2 Grain by-products

Dried brewers' grain

The extraction of malt from barley grain, produce brewers' grain which is the remaining residue and used as a by-product in dairy cow nutrition (Griffiths, 1971). The CP content is about 26% with a well-balanced good AA composition (Dhiman *et al*., 2003). Dried brewers' grain used as a protein source resulted in better performance and improved efficiency compared to soybean meal and urea in a study using beef calves (Murdock *et al*., 1981).

Corn gluten meal and corn gluten feed

Two valuable protein products result from the wet-milling industry of maize. Following the removal of starch, fibre and germ from maize and separation of the bran, the residue is dried to produce corn gluten meal with a 60% CP content. (Blasi *et al*., 2001). Corn gluten meal is also known as prime gluten. The bran that is removed, also contains some gluten protein

and is known as corn gluten feed or gluten 20, as it contains 20% CP. High concentrations of prolamins and glutelins protect the protein to a certain extent from ruminal degradation, whereas the favourable methionine:lysine ratio of corn gluten meal ensures a high quality RUP. Inconsistent milk production has been reported in some studies when corn gluten meal replaced soybean meal. The increased milk production was the result of the high RUP, whereas decreased milk production was caused by insufficient RDP or an EAA imbalance (Imran *et al*., 2018). Another product, corn gluten meal 40, is a mixture of corn gluten meal 60 and corn gluten feed (containing hulls and germ).

Distillers grain

During the production of ethanol, used in beverage liquor and for fuel, distillers grain becomes available as a co-product. The production process is done by fermentation of grains to alcohol, whereby further processing takes place to form the co-products (Curzaynz-Leyva *et al*., 2019). Dried distillers grain (DDG) and dried distillers grain with solubles (DDGS) are the most common of these co-products that are used in animal nutrition. The RUP content of DDG is around 55% of the CP and that of DDGS around 47%. A high intestinal digestibility has also been reported (Linn *et al*., 1996).

2.6.2 Animal proteins

2.6.2.1 Fishmeal

Fishmeal is an excellent source of protein for production animals. The primary product is fresh fish being cooked, pressed, dried and milled to get rid of most oil and water. In the case of brown fish meal, the fish used to produce it is not in demand by humans. Fishmeal in animal diets showed improvement in fertility, forage utilization, milk output and health. Protein digestibility can be as high as 95% in well-processed fish meals but when too much heat is used in processing, the protein digestibility can be as low as 60% (McDonald *et al*., 2011). Fishmeal consists of at least 60% CP, with a RUP of 60 to 70%. Fishmeal is one of the best AA balanced protein feedstuffs that can be provided to cows for growth and milk production. High calcium and phosphorus levels are also found in fishmeal (Guthrie and West, 1991). Fishmeal is also a good source of the vitamin B complex, especially choline, vit. B_{12} and riboflavin (McDonald *et al*., 2011). According to the RSPCA, a maximum inclusion level of 10% of total dietary DM is recommended. Adaptation should be applied slowly, because of the palatability and odour (Guthrie and West, 1991). The usage of fishmeal fluctuates, because of availability, price and the product mix. The price of fishmeal is high, but it is still one of the best protein sources available (DAFF, 2017).

2.6.2.2 Blood meal

Blood meal, obtained from abattoirs as a by-product, is a high-quality protein source. The product is dried (spray, batch and ring) before use and is well known for the high degree of RUP it provides. Only 20% of the 87% CP is degraded in the rumen. Lysine, arginine, methionine, cysteine and leucine are supplied by blood meal (McDonald, 2011). If excessive heat is applied during the drying process, the protein will be damaged and digestibility will be poor, resulting in an inferior quality product. Blood meal is mostly included in diets of lactating cows for production, because of the supply of limiting AA, especially lysine. Recommendations are not to exceed 300g per cow per day. When this amount is exceeded, palatability and milk fat may be reduced (Western Dairy Science Inc, 2004).

2.6.2.3 Meat and bone meal

Meat and bone meal is a rendered product from mammal tissues and bones. Calcium and phosphorus levels are high, because of the bone inclusion (Guthrie and West, 1991). It contains a minimum of 4% of phosphorus and the calcium level is usually not more than 2.2 times that of phosphorus. The CP content varies between 50 and 55% depending on the amount of bone included and approximately 50% of the CP is rumen degradable (Harris *et al*., 2003). Because of the possible contamination of bone with nervous tissue, meat and bone meal may not be fed to cattle so as to prevent any possibility of spreading BSE in cattle (Tisch, 2006). Therefore, non-cattle sources should be used in cattle diets (Shaver, 2001). The processing procedure may cause variation in CP levels, digestibility and AA composition (Bozkurt *et al*., 2004).

2.6.2.4 Feather meal

Hydrolysed poultry feather meal is obtained from steam cooking under pressure of clean, undecomposed feathers. Raw feathers are indigestible because the protein is in the form of keratin. Hydrolysation breaks the keratin bonds and renders the product quite digestible. Despite the high CP content of up to 85%, the protein quality is regarded as only fair due to the suboptimal amino acid profile (Tisch, 2006). According to McDonald (2011), histidine and lysine are the first limiting amino acids in feather meal. Palatability is usually not a problem. According to Guthrie and West (1991), the RUP content is almost 70% RUP, but it should not be included at more than 10% of a concentrate mix (Guthrie and West, 1991).

2.7 General aspects of protein sources

The most important characteristics of protein sources are summarised in Table 2.2.

Protein source	**N**	**RUP (% of CP) Avg ± SD (Range)**	**ID (% of RUP) Avg ± SD (Range)**	**IADP AVG ± SD (Range)**
Plant proteins				
Cottonseed meal, solvent	1	46	71	33
Cottonseed meal, mechanical	1	55	80	43
Soybean meal	5	25±3 (22-29)	90±4 (86-93)	22±2 (20-25)
Soybean meal, expeller	6	47±6 (38-53)	93±7 (83-100)	44±3 (38-53)
Soybean meal, non-enzymatically browned	6	66±8 (57-77)	88±4 (82-92)	58±7 (49-67)
Grain by-products				
Brewers grain, dried	5	57±5 (50-63)	77±2 (73-79)	44±5 (37-49)
Corn gluten meal	2	83±2 (82-85)	89±4 (86-91)	74±5 (70-77)
Distillers grains, dried	5	56±8 (47-64)	81±5 (72-85)	46±8 (36-53)
Animal proteins				
Blood meal, batch-dried	12	88±6 (78-98)	63±17 (29-86)	55±14 (25-75)
Blood meal, ring-dried	10	83±4 (76-89)	81±6 (72-90)	67±7 (58-76)
Feather meal, hydrolysed	12	76±11 (50-88)	67±6 (58-75)	51±9 (36-64)
Fish meal, Menhaden	13	65±4 (59-73)	80±5 (73-88)	52±4 (43-57)
Meat and bone meal	11	59±13 (40-88)	55±10 (41-70)	33±10 (21-56)

Table 2. 2 Rumen undegradable protein (RUP), intestinal CP digestion (ID) and intestinally absorbable dietary protein (IADP) of important protein sources (Stern *et al*., 2006).

2.8 Conclusion

Formulating dairy cattle diets to meet protein requirements for dairy cows has shifted from formulating for CP alone to that of metabolisable protein which is digested and absorbed as AA in the small intestine. The MP is derived from RUP, microbial protein and endogenous protein, but a fine balance between RUP and RDP is required for optimal microbial protein synthesis and animal production. Optimising diet formulation would also result in more space for other raw materials. In this review, attention was given to protein digestion and metabolism, ways to manipulate protein degradability and different protein sources available for animal production. From the literature, it appears that there is need for research on protein binders to decrease ruminal protein degradability of oil cake meals and the effect thereof on intestinal digestibility.

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Chapter 3

The effect of Bioprotect® on *in sacco* and *in vitro* protein degradation

Abstract

The objectives of the study were to determine the effect of a protein binder on in sacco *DM and CP degradability, as well as effective CP degradability on protein feedstuffs commonly used in dairy cow diets in South Africa. An* in vitro *trial was also done where the same protein sources were incubated for 0, 4, 8 and 16 h to compare CP degradation with values obtained in the in sacco trial. Soybean, sunflower and canola oilcake meals were identified as the protein sources. Oilcake meals were ground through a 1 mm screen and then sieved through a 106 µm sieve. Feedstuffs weretreated with either distilled water or Bioprotect® at a rate depending on the CP content of the oil cake meal. The application rate was equivalent to 0.5L for each 1% CP in the substrate per tonne. In the in sacco trial, the treated feedstuffs were weighed out in a series of dacron bags for ruminal incubation of 0, 2, 4, 8, 16, 24 and 48 hours. Four ruminally cannulated lactating Holstein cows were used as donorsof rumen liquid for the in sacco trial. Bioprotect® increased the a-value (0 h incubation) of all the substrates, indicating a higher water solubility following treatment. In the in sacco trial, Bioprotect® did not lower DM or CP degradability for any of the substrates. As a result of the higher a-values obtained with Bioprotect® treatment, the effective CP degradability (eDegCP) of all the substrates increased. The respective eDegCP values obtained with the Bioprotect® and Control treatments were 24.6 and 13.2% for soybean oilcake (P = 0.018), 59.4 and 45.4% for sunflower oilcake (P = 0.005) and 44.9 and 33.1% for canola oilcake (P < 0.001). In the* in vitro *trial, treatment*time interactions showed that Bioprotect® decreased CP degradation of canola meal after 8 h (49.3 vs. 54.2% for Control); P= 0.022) and 16 h of incubation (63.3 vs. 67.5% for Control; P = 0.04). For soybean meal, treatment also tended (P = 0.06) to decrease 16 h CP degradability (72.6 vs. 77.9% for Control). It was concluded that Bioprotect® appears to increase CP solubility, but the effect of treatment on CP degradability was not conclusive due to different tendencies observed in the in sacco and*in vitro*trials. However, the difference between treatments in the magnitude of* in vitro *CP*

degradability observed from 4 to 16 h suggested that Bioprotect® may indeed have a depressing effect on the degradability of the potentially degradable fraction but this effect may be shadowed by the increase in the soluble fraction observed in the Bioprotect® treatment. Due to the significant impact of the soluble fraction on the calculation of effective degradability, the end result is a higher effective CP degradability observed in the Bioprotect® treatments.

3.1 Introduction

Rumen microbes play an essential role in fulfilling nutrient requirements of the ruminant host, both in terms of fermentation end products in the rumen and by delivering a significant part of the metabolizable protein in the duodenum. Nitrogen requirements of the rumen microbes can be met by ammonia, but the host's organs and tissues need to be supplied with AA's. The AA's are provided via rumen undegradable protein (RUP) and microbial protein. This has resulted in systems being developed to determine the ruminal degradability of dietary protein in order to estimate RUP that enter the duodenum and can be digested to AA's for absorption from the small intestine (Walli *et al.*, 1993).

The feed protein fraction that escapes degradation in the rumen and passes to the small intestine is especially beneficial for high-yielding cows and increases profitability (NRC, 2001). The requirement of RUP is the difference between the total protein requirement by the host animal for milk production and the protein supplied by microbes. Low producing cows have low RUP requirements therefore microbial protein can supply most of the cows' total protein requirements (De Ondarza, 2004).

Meeting the daily nutrient requirements of dairy cows would result in optimum production. To meet these requirements the ruminal degradation of each feedstuff in the diet must be estimated to enable formulating a diet correctly. (Mohamed and Chaudhry, 2008).

Accurate measurement of ruminal nutrient degradability is required for use in feeding systems. In the current trial, both *in sacco* and *in vitro* methods have been used, but each method has its own set of problems. In the *in sacco* method, feed samples are placed in polyester dacron bags and inserted into the rumen of cannulated cows to determine the degradability of a specific nutrient in a feedstuff by measuring the disappearance of, for example, DM, NDF or N. The *in vitro* technique is a convenient, easier and less expensive alternative (Mohamed and Chaudhry, 2008), while *in vivo* techniques are expensive and require more labour to estimate intestinal digestion of protein (Stern *et al.*, 2007).

Oilcake meals contain high levels of CP, but they are highly degradable in the rumen. Fishmeal is higher in CP than oilcake meals and it has a high RUP content, but it is very expensive. Different treatments to oilcake meals could potentially increase the RUP value, which would increase milk yield of high producing dairy cows. Denaturing the protein of a feedstuff by heat treatment could produce a less rumen digestible feedstuff, but too much heat would result in a significant maillard reaction that would render the protein indigestible in the small intestine (De Ondarza, 2004). Over-processing with chemicals, such as formaldehyde, can also reduce nutritional value and protein digestibility in the small intestine.

The objective of the current trial was:

- To determine the effect of a potential protein binder on DM and CP disappearance parameters using *in sacco* and *in vitro* methods.

3.2 Materials and methods

3.2.1 Animals

Four ruminally cannulated lactating Holstein cows, 137 ± 18 (SE) DIM and weighing 713 ± 11 (SE) kg, were used in the trial. The cows were housed at the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa. The animals were kept in a free stall barn with the rest of the milking herd. Cows received a TMR twice daily (7h00 and 16h00). All procedures carried out in the current trial were approved by the Research Ethics Committee: Animal Care and Use at Stellenbosch University (protocol reference number AUC-2018-6802).

3.2.2 Treatments

The protein feedstuffs used in this study were three different oilcake meals, namely soybean, sunflower and canola. Each feedstuff was milled using a hammer mill (Scientic, RSA) with a 1 mm screen. The residues were sieved through a 106 µm screen with a brush to remove fine particles which could pass through the dacron pores undegraded. The residues on the top half of the sieve were stored in a cold room (4˚C) in plastic containers until used.

Two treatments were used on the samples for both the *in sacco* and *in vitro* trials:

1. Bioprotect®
2. Distilled water (Control)

Treatment of substrates with Bioprotect® and distilled water was done by spraying the relevant solutions on the substrates at a rate equivalent to 0.5 L per 1% CP per tonne. For

example, if soybean oil cake contains 47% of CP, the application (Bioprotect® or water) would be equivalent to 23.5 L/tonne

3.2.3 Procedure

3.2.3.1 In sacco

The dimensions of the dacron bags used in the trial were 100 mm x 200 mm with a nominal mean pore size of 53 µm. The bags were numbered with a permanent marker and dried at 50˚ C for 24 hours in a forced draught oven. After drying, the bags were cooled in a desiccator for 30 minutes before weighing. For each oil cake meal and treatment, an amount of 8 g of the treated substrate was accurately weighed and transferred to the bags. The open end of each bag was folded over twice before folding the seam in a tobacco bag manner and tying with a cable tie. The bags were placed into ladies' opaque stockings (size large to hold 6 bags per stocking). Each bag was separated with a knot in the stocking, in an order to ensure that each numbered bag was retrieved at the correct incubation time. Each stocking had a large glass marble (35 mm diameter) in the toe end, to add weight to ensure that the stocking would be kept down into the rumen fluid.

So-called catcher stockings were tied to the inside of the cannula lid and the stockings containing the Dacron bags were tied to the catcher as explained by Cruywagen (2006). The catchers were also marked with cable ties of different colours to facilitate treatment recognition when bags are retrieved. Six stockings (3 oilcakes, 2 treatments) were incubated in the rumen of each cow. The bags were inserted at 14:00 and retrieved after the following incubation times: 2 h, 4 h, 8 h, 16 h, 24 h and 48 h (NRC, 2001). A 0 h incubation time was also included where the bags were not incubated in the rumen but washed in cold water according to the NRC (2001) procedure. The 0 h incubation represents the a-value (soluble and rapidly degradable fraction) in the first order model that was used for data processing and analysis.

The collected bags were washed under tap water until the water ran clear and then placed in ice water for 15 minutes. The bags were then gently squeezed and dried between sheets of paper towel before they were stored in a cold room (4˚C). After retrieval of the last bags (48 hours) all the bags were washed in a washing machine using a gentle cycle setting according to the NRC (2001) protocol. After spin drying, the bags were dried at 60˚C for 48 hours in a forced draught oven.

3.2.3.2 In vitro

Dacron bags were prepared in the same way as for the *in sacco* trial with 8 g of substrate per bag, but instead of ruminal incubation, the bags were incubated in jars using the ANKOM Daisy^{II} Incubator (ANKOM Technology, New York).The incubation medium per jar consisted of 200 ml of rumen fluid as microbial inoculant and 800 ml of the Goering and Van Soest buffer (Goering and Van Soest, 1970), the composition of which is presented in Table 3.1. Rumen fluid was collected from the same cows that were used for the *in sacco* trials. After addition of the rumen fluid and before the incubation started, each jar was gassed with CO_2. A number of eighteen bags were incubated per jar and the trial was done in two runs. Four jars were used per run which included one jar per cow per treatment, thus using two cows per run resulting in four replications. In the second run, two different cows were used as rumen liquid donors, thus using the same four cows that had been used in the *in sacco* trial. The Daisy incubator was located in a heat controlled warm room at 39°C. After incubation, bags were retrieved and treated in the same way as the bags from the *in sacco* trial explained above.

3.2.3.3 Rumen fluid collection

Rumen fluid was collected in the morning before 07h00 on the day of incubation. Ethical clearance was obtained from Stellenbosch University's Animal Ethics Committee (Protocol reference number AUC-2018-6802).

Before collection, 1 L thermos flasks were filled with boiling water to preheat the flasks. Cows were put in a crush for rumen fluid collection. After removing the rumen cannula plugs, they were put into a bucket of hot water to become more flexible for easier replacing into the cannula opening. Rumen fluid was collected from different parts in the rumen and strained through two layers of cheesecloth into the flask through a funnel. Enough fluid was removed to fill the flasks to the brim to keep the content anaerobic. The cannula plug was then replaced into the cannula opening.

In the laboratory, the rumen fluid of each flask was strained through another four layers of cheesecloth into 2 L glass Erlenmeyer flasks that were preheated (39˚C). After straining, the pH was recorded, and each flask was gassed with CO_2 before taking them to the warm room (39°C) where the incubations were done.

3.2.3.4 In vitro incubation medium

The incubation medium (Goering and van Soest, 1970) was prepared according to the method of Goering and VanSoest, (1970) as indicated in Table 3.1. 800 ml of the medium

was measured into each incubation jar. Following addition of the reducing solution, the jars were gassed with CO_2 and taken to the incubation room (room temperature of 39°C). The rest of the procedure is discussed above.

Table 3.1 Reagents and quantity of the in vitro solutions (Goering and Van Soest, 1970).

Reagent	Quantity
Buffer solution:	
Distilled water (dH_2O)	1000 ml
Ammonium bicarbonate (NH_4HCO_3)	4 g
Sodium bicarbonate ($NaHCO_3$)	35 g
Macromineral solution:	
Distilled water (dH_2O)	1000 ml
Di-sodium hydrogen orthophosphate (Na_2HPO_4) (anhydrous)	5.7 g
Potassium dihydrogen orthophosphate (KH_2PO_4) (anhydrous)	6.2 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.6 g
Micromineral solution:	
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	13.2 g
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	10 g
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1 g
Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	8 g
Incubation medium:	
Distilled water (dH_2O)	1600 ml
Tryptose	8 g
Micromineral solution	400 µl
Macromineral solution	800ml
Rezasurin	4 ml
Reducing solution:	
Flask 1: Distilled water (dH_2O)	80 ml
Cysteine hydrochloride ($C_3H_7NO_2HCl$)	1 g
Potassium hydroxide pellets (KOH)	40
Flask 2: Distilled water (dH_2O)	80 ml
Sodium sulphide nonahydrate (NaS)	1 g

3.2.4 Chemical analysis

After drying the bags at 60˚C for 48 h, they were cooled for 30 minutes in a desiccator and weighed. Bags were then opened and emptied into small bottles to collect samples for N determination.

A Leco FP-528 was used to determine the N content according to the AOAC (2002) protein (crude) in animal feed and pet feed protocol. The N content of the original feedstuffs before treatment and the residue of each bag after incubation, drying and weighing was determined. The N percentage was multiplied by 6.25 to determine the CP content of each sample (AOAC, 2002). Before calculating disappearance values, the final DM content of all the substrates and residues was determined by drying samples at 105°C for 24 h. The ash content was determined according to the AOAC (2002) official method 942.05 (ash of animal feed).

3.2.5 Data analysis

The Solver function of Microsoft Office Excel was used to fit the DM and CP disappearance data according to a non-linear model (Ørskov and McDonald, 1979):

$$Y = a + b\,(1 - e^{-ct})$$

where Y = degradation at time t

a = soluble and rapidly degradable fraction

b = fraction that will potentially be degraded over time

c = rate of degradation of fraction b

e = natural logarithm

The effective CP degradability was calculated as follows:

$$D_{eff} = a + (bc / c + k_p)$$

where D_{eff} = effective degradability

a,b and c = degradability parameters determined with the non-linear model

k_p = passage rate from the rumen

The effective degradability includes k_p because passage rate from the rumen affects degradation.

Data were analysed according to a main effects ANOVA using Statistica 10 (2011). The main effects were treatment, time and cow. For the *in vitro* trial, repeated measures analysis was done to determine the CP disappearance over time, because there were not enough incubation times to determine non-linear parameters. In all cases, significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

3.3 Results and discussion

The chemical composition of the raw materials used in the study is shown in Table 3.1. The three oilcake meals differed mainly in protein content being higher in soybeans in comparison to sunflower OCM and canola OCM. . Because the level of Bioprotect® treatment depends on the CP content of the feedstuff, it follows that the higher the CP content is, the more Bioprotect®would be required for treatment.

Table 3.2 The chemical composition of the protein sources used in this study.

	SBM (%)	SFM (%)	CM (%)
DM	89.4	91.4	88.8
CP	47.0	30.5	34.6
Ash	7.8	5.6	8.0

SBM = soybean oilcake meal, SFM = sunflower oilcake meal, CM = canola oilcake meal, DM = dry matter, CP = crude protein

3.3.1 *In sacco* DM degradability

In sacco DM disappearance results are presented in Table 3.3. Trend lines (Figure 3.1) were fitted using the parameters derived from the non-linear function.

3.3.1.1 Effect of Bioprotect® treatment on a-values for DM degradability

The a-values (soluble and rapidly degradable fraction) of all three protein sources (Table 3.3) differed between treatments, with Bioprotect® resulting in higher values. Because the samples were sieved through 106 µm before treatment, the values and differences between treatments cannot be ascribed to fine particle washout. The higher a-values observed for Bioprotect® would suggest that this treatment increased water solubility of nutrients, including protein.

It was expected that the Bioprotect® treatment would result in lower a-values as Bioprotect® seems to bind to the amino groups of proteins, thus protecting them against degradation. However, because the a-values represent the 0 h values (water washing), these values do not reflect ruminal degradation. It should, however, be kept in mind that the DM in the oilcakes contained more nutrients than protein alone and that the effect of treatment on DM a-values involves various nutrients. Although the effect between the treated and control means was unexpected, it was similar for soybean and canola oil cake meal but not for sunflower oilcake meal. Nel (2012) reported higher a-values (34 and 33%) than those in the current study for similarly sieved soybean and sunflower meal. In the current study, the increase observed in the soluble fraction after Bioprotect® treatment cannot be readily explained. The results of further investigations on the solubility of the treated feedstuffs are discussed in Chapter 5.

Table 3.3 The effect of Bioprotect® treatment of different protein sources on in sacco ruminal DM degradation parameters in lactating Holstein cows.

	Treatment			
Oilcake meal	Bioprotect®	Control	SEM	*P*
Soybean				
a[1]	24.9	14.0	0.489	< 0.001
b	70.9	71.1	2.839	0.968
c	0.033	0.032	0.001	0.108
effDeg[2]	45.7	34.2	0.818	0.002
Sunflower				
a	5.5	2.4	0.446	0.02
b	42.4	47.1	0.916	0.035
c	0.09	0.08	0.005	0.357
effDeg	27.8	26.5	0.339	0.05
Canola				
a	11.0	6.5	0.237	< 0.001
b	50.6	48.8	1.237	0.382
c	0.06	0.05	0.005	0.179
effDeg	32.8	25.5	0.968	0.013

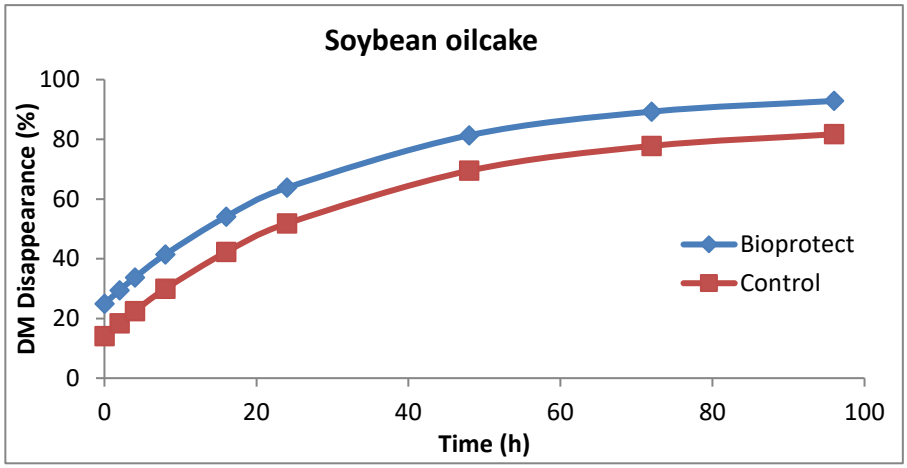
SEM = Standard error of the mean.

[1]a = soluble and rapidly degradable fraction; b = potentially degradable fraction; c = rate at which b is degraded

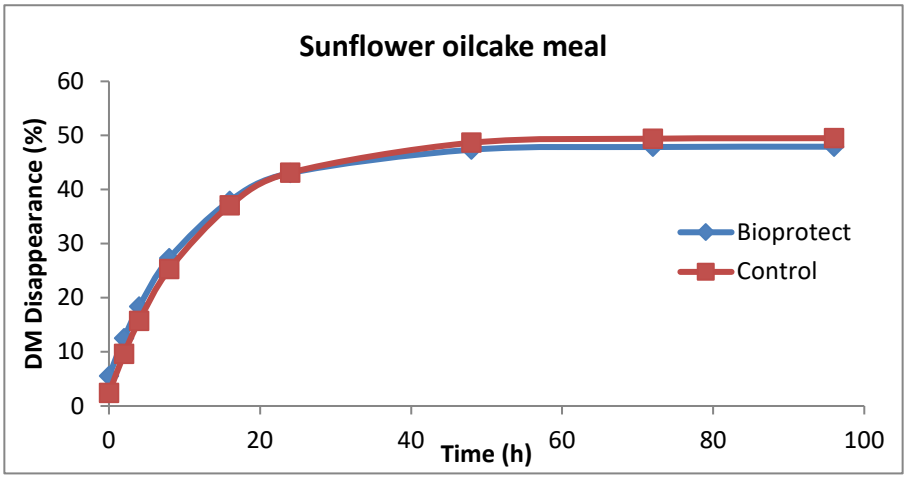
[2]effDeg = effective degradability (k_p = 0.08)

Rates and extent of DM degradation are shown in Figure 3.1 (A-C).

(A)



(B)



(C)

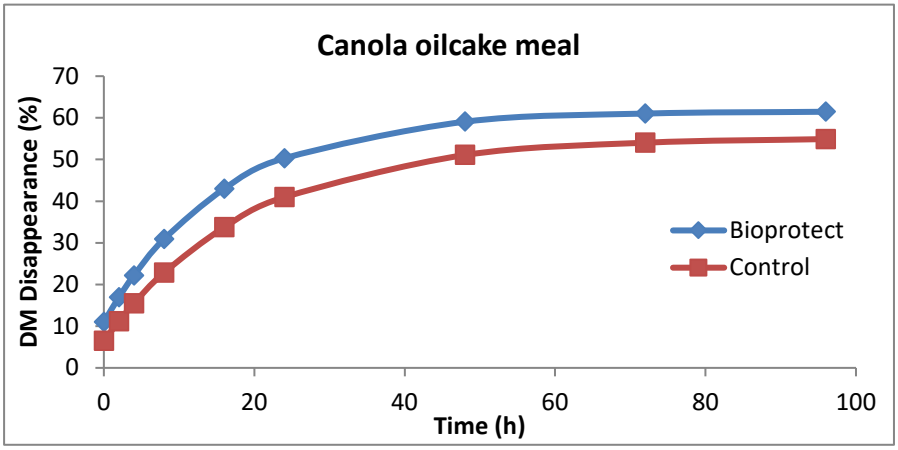


Figure 3.1 (A-C) The effect of Bioprotect® treatment of different protein sources on *in sacco* DM disappearance over time.

3.3.1.2 Effect of Bioprotect® treatment on b-values for DM degradability

The potentially degradable DM fraction for soybean and canola oilcake meals showed no significant differences between the two treatments. The b-values of sunflower oilcake meal, however, differed significantly ($P<0.05$) between treatments, with the mean value of Bioprotect® being lower than that of the control group. Although the difference was significant ($P = 0.035$), it was small and in Figure 3.1 (B) it can be seen that treatment effects only start to become apparent after around 40 hours of incubation. Griffiths (2004) reported that extrusion increased the DM b-fraction in soybean, canola and sunflower oil cakes.In a study by Nel (2012) who used similarly sieved oilcakes, similar b-values were reported forsoybean meal (66%) and sunflower meal (40%).

The discussion of DM disappearance alone cannot lead to a final conclusion, but it may indicate that material can potentially be protected by Bioprotect®. Results on CP disappearance are discussed later.

3.3.1.3 Effect of Bioprotect® treatment on c-values for DM degradability

There was no significant difference between treatments in any of the three protein sources for the rate at which b-value (potentially degradable fraction) was degraded. Table 3.1 shows that the consistent numerical difference between treatments was as small as 0.001 and 0.01 for the three oilcake meals. In the non-linear model that was used to solve the degradability estimations, the different parameters (a-, b- and c-values) are interdependent and the one affects the other. For example, a high a-value, as well as a high b-value, would result in a high c-value, and vice versa. This result in some marked differences were observed between different studies. Nel (2012), for example, reported much higher c-values for soybean meal (0.078) and sunflower meal (0.148) than what were observed in the current study, due to the higher a- and b-values reported in that study.

3.3.1.4 Effect of Bioprotect® treatment on effective DM degradability

The passage rate of feed from the rumen of lactating dairy cows (k_p) is accepted to be 8% per hour, and as passage rate and rumen retention time are reciprocals of each other, the mean rumen retention time for $k_p = 0.08$ is calculated to be 12.5 hours. Significant differences were observed (Table 3.1) for all three oilcakes where Bioprotect® resulted in higher effective degradability (effDeg) values compared to Control. The difference between Treatment and Control was much smaller for sunflower oilcake than for soybean and canola, which was expected as the b-value of sunflower oilcake was significantly lower. Because

effective DM degradability does not really have practical value, no documented results were found to compare with the current study.

3.3.2 *In sacco* CP degradability

In sacco CP disappearance results are presented in Table 3.4. Curves (Figure 3.2) were also constructed using the parameters derived from the non-linear function.

Table 3.4 The effect of Bioprotect® treatment of different protein sources on in sacco ruminal CP degradation parameters in lactating Holstein cows.

	Treatment			
Oilcake meal	Bioprotect®	Control	SEM	*P*
Soybean				
a[1]	8.9	0.5	1.224	0.017
b	86.3	82.7	5.224	0.661
C	0.019	0.015	0.001	0.124
effDeg[2]	24.6	13.2	1.722	0.018
Sunflower				
a	12.4	7.2	1.062	0.042
b	76.3	80.2	1.412	0.142
c	0.133	0.076	0.005	0.004
effDeg	59.4	45.4	0.592	0.005
Canola				
a	16.4	6.2	0.634	0.002
b	65.9	55.2	5.022	0.232
c	0.061	0.087	0.015	0.318
effDeg	44.9	33.1	0.554	<0.001

SEM = Standard error

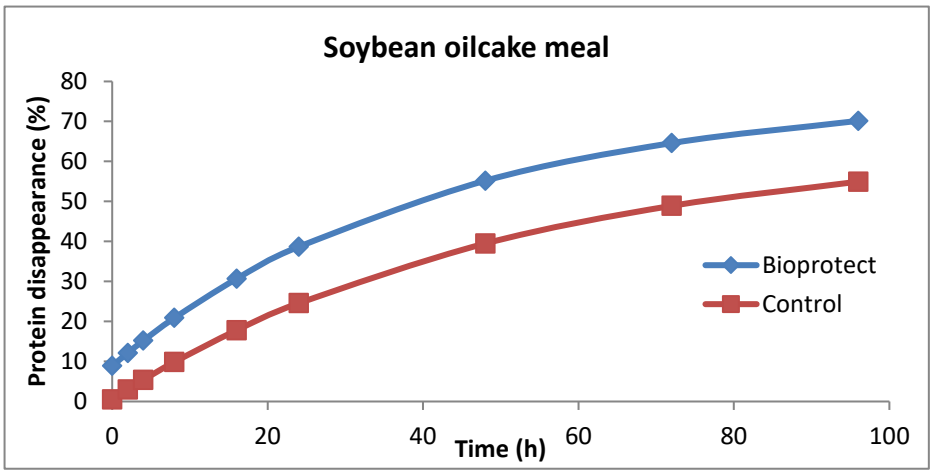
[1]a = soluble and rapidly degradable fraction; b = potentially degradable fraction; c = rate at which b is degraded

[2]effDeg = effective degradability (k_p = 0.08)

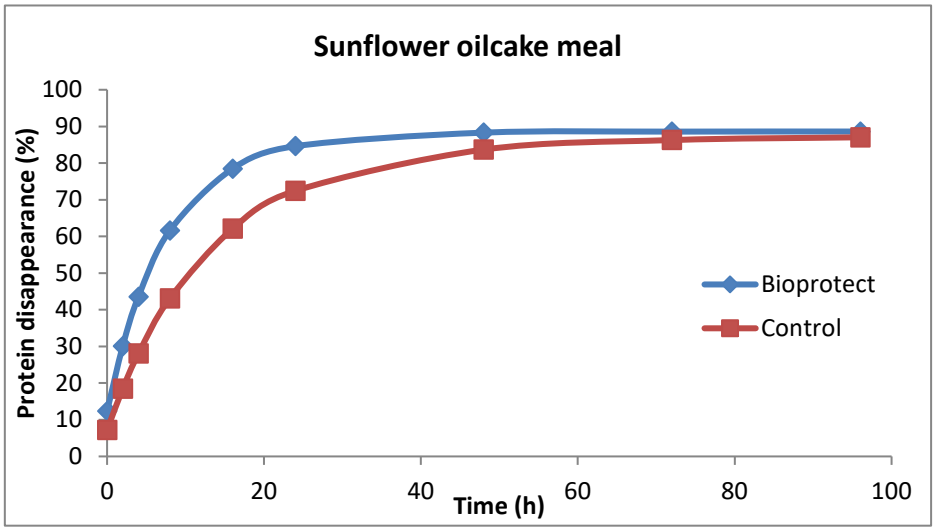
3.3.2.1 Effect of Bioprotect® treatment on a-values for CP degradability

The a-values of all three protein sources (Table 3.2) were higher with the Bioprotect® treatment compared to the Control. As mentioned previously, the samples were sieved through 106 µm before treatment, and the differences between treatments can thus not be ascribed to fine particle washout. The higher a-values observed for Bioprotect® would suggest that this treatment increased the water solubility of proteins.

(A)



(B)



(C)

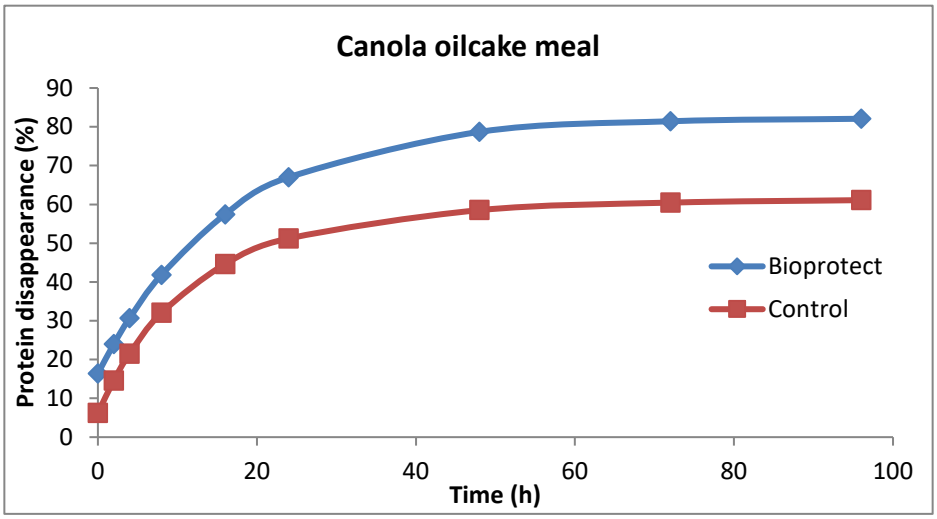


Figure 3.2 The effect of Bioprotect® treatment of different protein sources on *in sacco* CP disappearance over time.

For reasons mentioned previously, it was expected that the Bioprotect® treatment would result in lower a-values, but because the a-values (0 h) represent the water washing phase, these values do not reflect ruminal degradation. As in the case of DM, the difference between treatments was consistent for all the oilcakes. Erasmus (1988) reported an a-value of 10% for unsieved soybean meal and 46% for sunflower. Nel (2012) reported a-values of 30.5% for unsieved soybean meal and 16.3% for 106 µm sieved samples. Corresponding values for sunflower meal were 37.3% (unsieved) and 130.5% (sieved). According to Griffiths (2004) extrusion (compared to untreated) decreased the a-value of CP in soybean and canola oilcakes but increased it in the case of sunflower oilcake. It can be expected that different treatments and different batches of the same type of feedstuff may have different effects on CP solubility. In the current study, the reason for higher a-values after Bioprotect® treatment are unclear and results of further investigations on the solubility of the treated feedstuffs are discussed in Chapter 5.

3.3.2.2 Effect of Bioprotect® treatment on b-values for CP degradability

All three protein sources showed no significant b-value differences between Bioprotect® and Control. As it was expected that Bioprotect® would provide protection against CP degradability, the current results were unexpected. In a study by Griffiths (2004), extrusion increased the b-values of soybean and sunflower oilcakes but had no effect on canola oilcake. Nel (2012) sieved samples of protein sources through different sieve sizes to remove fine particles that could be washed out of dacron bags without being digested. She reported that whereas sieving decreased the a-values it increased the b-values of soybean and sunflower meals from 71.1% to 88% (soybean meal) and from 55.9% to 62.3% (sunflower meal). As in the case of treatment effects on solubility, it appears that different treatments and batches have different effects on the potential CP degradability of protein sources.

The curves in Figure 3.2 show marked differences over time between the Control and Bioprotect® treatments for all three oilcakes. An asymptote was not reached with soybean meal (A) which was still increasing at 100 hours. Final degradation values of soybean and canola meals after 96 h were higher in the Bioprotect® treatment compared to the Control, but values were quite similar in the case of sunflower meal. In sunflower meal, the higher rate of degradation can be observed in the Bioprotect® treatment.

3.3.2.3 Effect of Bioprotect® treatment on c-values for CP degradability

There was no significant difference between treatments in the rate of degradation for both soybean and canola oilcakes. However, sunflower oilcake meal was degraded significantly

faster in the Bioprotect® treatment. Since there were no differences between treatments in the c-values regarding DM, the current result was not expected. Extrusion had no effect on the CP degradation of soybean oilcake but lowered the rate in sunflower oilcake and tended to lower it in canola oil cake (Griffiths, 2004). Nel (2012) reported no effect of sieving in c-values of soybean and sunflower meals.

3.3.2.4 Effect of Bioprotect® treatment on effective CP degradability

Calculated at k_p = 0.08, which would be equivalent to a mean rumen retention time of 12.5 h, a significant difference in effDeg was observed between treatments for all three protein sources. Bioprotect® resulted in significantly higher effDeg values, which was the opposite of what was expected when the trial commenced. When protein sources were extruded, Griffiths (2004) observed significant decreases in effDeg. Literature values of effective CP degradability for soybean meal and canola meal are generally higher than those reported in the current study. Erasmus *et al*. (1988) reported effDeg values of 51.4% for soybean meal and 76% for sunflower meal, whereas Nel (2012) reported fairly similar values of 55.3% and 72% for the same oilcakes. According to Schroeder *et al*. (1995), effDeg was 73% for soybean meal. Susmel *et al*. (1993), using a k_p of 0.07, reported 52% for soybean meal and 76% for sunflower meal. Using a k_p of 0.06, Alexandrov (1998) found effDeg values of sunflower meal to be 74%. Although effDeg values obtained in the current study are lower than most reported in the literature, Biotin consistently increased effDeg of CP. For both treatments, sunflower oilcake had the highest effDeg values, followed by canola oilcake whereas soybean oilcake had the lowest values. This is consistent with the literature (Ha and Kennelly, 1984; Erasmus *et al*., 1988).

3.3.3 *In vitro* CP degradability

Following the *in sacco* trial, it was decided to do another trial to confirm the results which were contrary to what was expected. The effect of Bioprotect® treatment on the same three protein sources was thus evaluated again, but in an *in vitro* trial. *In vitro* trials require less labour and are less expensive than *in sacco* trials (Holden, 1999). In the *in vitro* trial, the effect of treatment was determined on CP degradability only, and samples were incubated for 4, 8 and 16 h.

Results are presented in Table 3. 5 and Figure 3.3 (A to C).

Table 3.5 The effect of Bioprotect® treatment of three protein sources on in vitro CP degradation at various time points. Values are LS means expressed as % of CP.

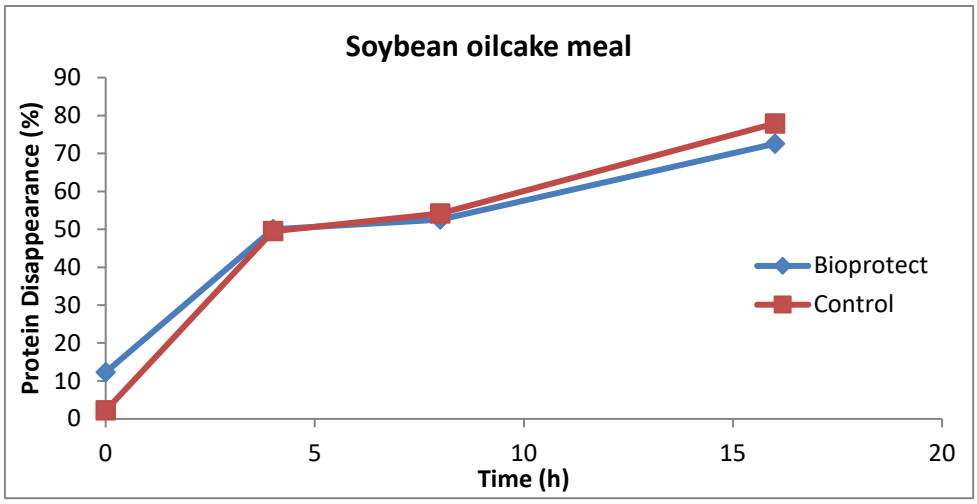
Oilcake meal	Time	Treatment		P-value
		Bioprotect®	Control	
Soybean	**0**	12.3	2.2	< 0.001
	4	50.0	49.5	0.832
	8	52.6	54.2	0.536
	16	72.6	77.	0.060
Sunflower	**0**	17.1	3.4	< 0.001
	4	74.2	71.6	0.078
	8	78.4	79.9	0.244
	16	88.7	89.3	0.681
Canola	**0**	15.0	7.4	< 0.001
	4	47.9	40.3	0.002
	8	49.3	54.2	0.022
	16	63.3	67.5	0.040

Although the magnitude of the 0 h values differed somewhat from the model derived a-values observed in the *in sacco* trial, the soluble and rapidly degradable fraction was again significantly higher for the Bioprotect® treated oilcakes (Table 3.5). In the control treatment of a study by Griffiths (2004), much higher a-values (comparable to 0 h in the current study) were reported for canola, namely 70% which is unrealistic and 29% for sunflower meal. Nel (2012) reported 11.5% for soybean meal and 27.4% for sunflower meal. The higher CP degradability of the Bioprotect® treated oilcakes in the current study was still observed after 4 h for canola oilcake, while a tendency to this effect (P = 0.08) was observed for sunflower oilcake. In the case of soybean oilcake, there was no difference between treatments at 4h.

After 8 h of incubation, no treatment effects were observed for soybean meal and CP disappearance values were similar. The 8 h disappearance value obtained in the Control (54%) was lower than the 77% reported for Control by Griffiths (2004). After 8 h in the current study, CP degradation started to decrease for the Bioprotect® treatment and by 16 h

there was a strong tendency ($P < 0.06$) towards lower CP degradation in the Bioprotect® treatment.

(A)



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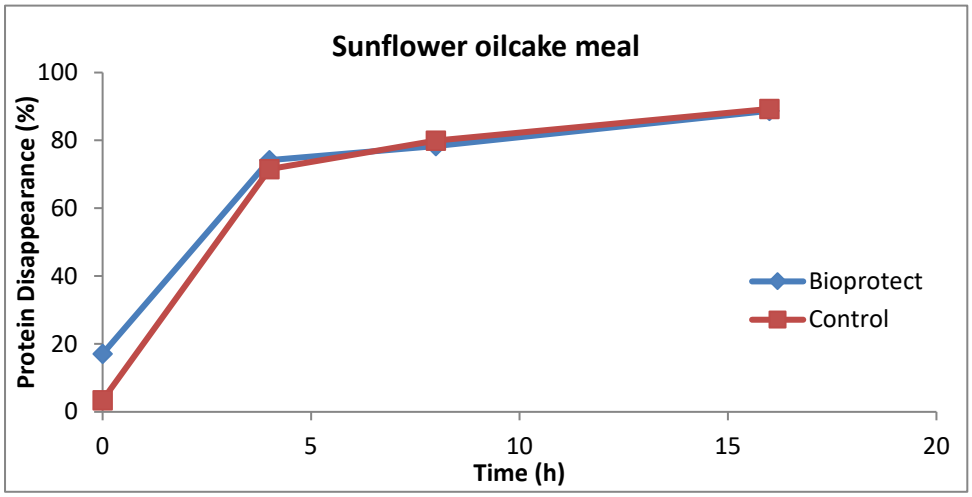

(C)

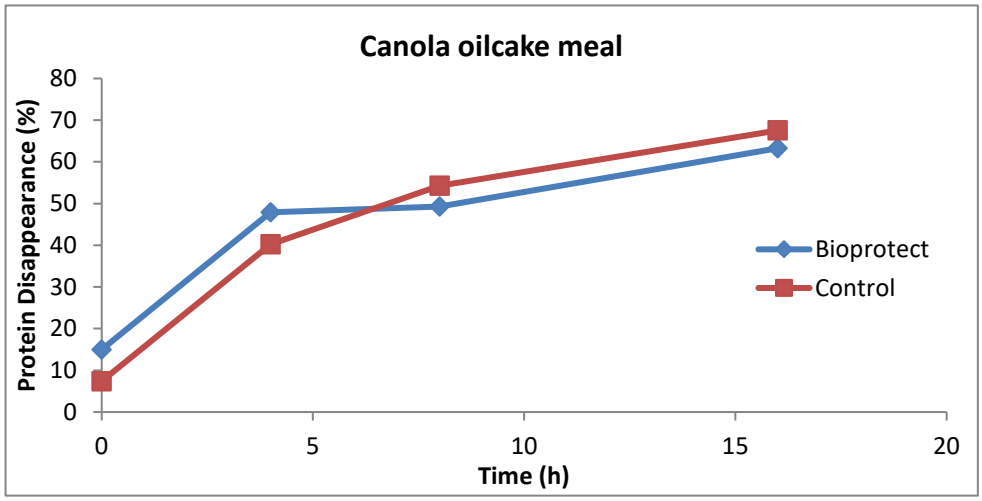

Figure 3.3 (A-C) The effect of Bioprotect® treatment of different protein sources on *in vitro* CP disappearance over time.

Considering that the soluble CP fraction of the Bioprotect® treated soybean meal was already 10 percentage units higher than the control, the degradation of soybean CP from that point onwards was in effect much lower than that of the Control (60% vs. 75%, respectively). This is also visible in Figure 3.3 (A). Nel (2012) reported 12 h CP disappearance values of soybean meal and sunflower meal to be 57% and 81.3%, respectively. These values are intermediate between 8 and 16 h values found in the current study.

In the case of sunflower meal, no treatment effects on CP degradability were observed from 8 to 16 h. As in the case of soybean meal, the degradation (disregarding the soluble fraction) of sunflower meal CP from 0 h to 16 h appeared to have been lower for the Bioprotect® treatment than for the Control (71.6% vs. 85.9%). This can be observed in Figure 3.3 (B).

Canola meal showed a similar tendency than the other oilcakes, but the effects were more significant (Table 3.5 and Figure 3.3 C). At 4 h the CP degradability was still higher in the Bioprotect® treatment, but between 4 and 8 h it appears that degradation started to slow down and by 8 h, CP degradation in the Bioprotect® treatment was significantly lower ($P = 0.02$) than in the Control. Again, when taking the soluble CP fraction into account, the net CP degradation after 0 h up to 16 h was less in the Bioprotect® treatment (48.3%) than in the Control (60.1%).

3.4 Conclusion

Bioprotect® increased the a-value (0 h incubation) of all the substrates, indicating a higher water solubility following treatment. In the *in sacco* trial, Bioprotect® did not lower DM or CP degradability for any of the substrates. As a result of the higher a-values obtained with Bioprotect® treatment, the effective CP degradability (eDegCP) of all the substrates increased. In the *in vitro* trial, treatment * time interactions showed that Bioprotect® decreased CP degradation in canola meal after 8 and 16 h of incubation and tended to decrease 16 h CP degradability in soybean meal. It was concluded that Bioprotect® appears to increase CP solubility, but the effect of treatment on CP degradability was not conclusive due to different tendencies observed in the *in sacco* and *in vitro* trials. However, the difference between treatments in the magnitude of *in vitro* CP degradability observed from 4 to 16 h suggested that Bioprotect® may indeed have a depressing effect on the degradability of the potentially degradable fraction but this effect may be shadowed by the increase in the soluble fraction observed in the Bioprotect® treatment. Due to the significant impact of the soluble fraction on the calculation of effective degradability, the result was a

higher effective CP degradability observed in the Bioprotect® treatments. The CP degradability of the soluble protein fraction is unknown and warrants investigation. This was the objective of a study that will be discussed in Chapter 5.

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Chapter 4

The effect of a protein binder on intestinal digestibility using the Ross assay

Abstract

*The objective of the study was to determine the effect of a potential protein binder (*Bioprotect®*) on the intestinal digestibility of three protein sources. The assay developed by Ross et al. (2013) was used to determine the intestinal digestibility of treated soybean, sunflower and canola oilcake meals (OCM). Each OCM was milled through a 4.5 mm screen before treatment. Treatment was done by spraying sub-samples of the OCM with either distilled water (Control) or* Bioprotect®*, both at a rate equivalent to 0.5 L/tonne for each 1% CP in the substrate. Rumen liquid was collected from six lactating Holstein cows, fitted with rumen cannulae. Rumen fermentation, gastric digestion and intestinal digestion were the three phases of the assay, where the first phase represents microbial digestion in the rumen and the last two phases represent intrinsic enzyme digestion. Incubation times were 12 hours for the rumen fermentation phase, 1 hour for the gastric digestion phase and 24 hours for the intestinal digestion phase. In this trial, the interest was in the total apparent digestibility values of all three phases, representing total intestinal digestibility (ID). No significant differences in ID were observed between the protein binder and the control group for all OCMs. Respective values for the protein binder and control treatments were 74.7 and 73.6% for soybean meal, 62.1 and 63.9% for canola meal, and 70.5 and 71.5% for sunflower meal. It was concluded that treatment of oil cake meals with the specific protein binder did not increase total intestinal digestibility.*

4.1 Introduction

The potential increase of milk protein and N efficiency can be the result of balancing dairy cow diets for AA. The available AA in the small intestine (SI) of cows are supplied by ruminal microbial CP, rumen undegradable protein (RUP) and endogenous protein (Paz *et al*., 2014). The intestinal digestibility (ID) of RUP is an important parameter determining milk production of dairy cows. Before the NRC (2001) published RUP digestibility values that range from 50% to 100%, all ID values of feedstuffs were accepted as 80%. Research showed, however, that the ID values of RUP from concentrates are higher than those derived from forages after 16 hours of rumen incubation (Wang *et al*., 2015). Concentrates undergo different processing methods and vary in terms of nutrient sources, whereas forages differ in terms of type and harvesting time (maturity), which would result in different digestibility percentages (Wang *et al*., 2015).

Dairy cow diet formulations are often based on feed library estimates. More accurate models are developed to predict the nutrient supply and nutrient balance, especially when nutrient values are known for the specific feedstuffs that are available. Diets are formulated to meet and not exceed the MP requirement, confirming the importance of accurate intestinal digestibility estimates of protein and AA's. A three-step assay to estimate *in vitro* intestinal digestibility values of protein feedstuffs used in ruminant diets was developed by Ross *et al*. (2013).

Methods used to determine ID of RUP include the original three step *in vitro* (OTS) method developed by Tilley and Terry (1963), the modified three step *in vitro* (MTS) method developed by Ross *et al.* (2013), the acid detergent insoluble nitrogen (ADIN) method described by Goering *et al.* (1970) and the widely used mobile nylon bag (MNB) method, as described by Ørskov and McDonald (1979). The last-mentioned method has many advantages including the significant correlation of the values with the *in vivo* method. Disadvantages were found for each method, for example time-consuming, expensiveness and environmental pollution (Wang *et al*., 2015). Sample loss and variation was eliminated with the development of the Ross assay (Ross *et al*., 2013).

The objective of the current trial was to determine the effect of a potential protein binder on the intestinal digestibility of soybean meal, canola meal and sunflower meal.

4.2 Materials and methods

4.2.1 Animals

Six ruminally cannulated lactating Holstein cows, 163 ± 20 (SE) DIM and weighing 728 ± 14 (SE) kg, were used for this trial. The same rumen fluid collection procedure was followed as the one discussed in Chapter 3.2.3.3. The cows were housed at the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa. The animals were kept in a free stall barn with the rest of the milking herd and received a lactating cow TMR twice daily (07h00 and 16h00). All procedures carried out in the current trial were approved by the Research Ethics Committee: Animal Care and Use at Stellenbosch University (protocol reference number AUC-2019-6802).

4.2.2 Treatments

Three oilcake meals (soybean, sunflower and canola) were used in this trial. Each oilcake was milled through a 4.5 mm screen using a laboratory hammer mill (Scientic, RSA) and samples were stored in honey jars in a cold room at 4˚C until used.

Oilcake meals were then treated with either distilled water (dH_2O) or Bioprotect®. Treatment was done by spraying the relevant solution at a rate equivalent to 0.5 L per 1% CP per tonne.

4.2.3 Preparations

The CSND was included in the assay as a fermentation control sample and for the estimation of microbial protein contamination. Maize silage samples were collected from a farm near Klipheuwel, Western Cape, RSA.

4.2.3.1 ND solution used to prepare CSND

The following were added to a 5 L glass beaker with a magnetic stirrer (20-40 cm): 93.05 g of EDTA, 34.05 g of sodium tetra borate, 22.8 g of disodium-hydrogen orthophosphate, 150 g of sodium lauryl sulphate and 3 L of distilled water. After addition of 50 ml of 2-ethoxyethanol to the glass beaker, the beaker was heated, and the contents stirred until all the salts were dissolved. The stirrer bar was removed, and the solution was transferred to a 5 L volumetric flask and filled with distilled water to the 5 L mark. The solution was thoroughly mixed before it was used.

4.2.3.2 Ammonium sulphate solution 1M

Distilled water (800 ml) and 132.14 g of ammonium sulphate, along with a 20 cm stirrer bar, were added to a 1 L glass beaker. Once the salt was dissolved, the stirrer bar was removed, and the solution transferred to a 1 L volumetric flask.

4.2.3.3 Preparation of CSND

The fresh silage was dried at 50˚C for 72 hours. The dried silage was milled through a 2 mm screen using a laboratory hammer mill (Scientic, RSA) and stored in a cold room (4˚C) until used. An amount of 120 g of dried maize silage and 120 g of sodium sulphite was mixed in a 5 L glass beaker with the addition of 3 L of ND solution, as well as 24 ml of heat stable amylase (Ankom Technology, Fairport, NY). The solution was boiled under a fume hood for 75 min and stirred occasionally. The contents were then washed with boiling water through a 106 µm sieve and placed on a tinfoil tray and dried overnight at 100˚C.

Because the resultant residue is only 20 to 25% of the originally weighed dry silage, the process had to be repeated until enough CSND (at least 15 g) was prepared. All the residues were milled through a 2 mm sieve using a laboratory hammer mill (Scientic, RSA). In the final step of CSND preparation, an amount of 15 g of the previously prepared CSND residue and 1 L of the ammonium sulphate solution (1M) were mixed together in a 2 L Schott bottle. The mixture was incubated at 39˚C overnight before the contents were washed three times through a 106 µm sieve with boiling water. Drying overnight at 100˚C was done before the residue was milled again through a 2 mm sieve and stored at 4°C until used.

4.2.3.4 Potassium phosphate buffer and enzyme mixture

The buffer was made up of two solutions. The first solution (A) was prepared in a 1 L volumetric flask with 500 ml of distilled water added. Then, an amount of 313.52 g of dipotassium phosphate (K_2HPO_4) was added and dissolved before filling to the mark with distilled water. The second solution (B) was prepared by dissolving 244.94 g of monopotassium phosphate (KH_2PO_4) in 500 ml of distilled water in a 1 L volumetric flask before filling to the mark. Volumes of 875 ml of solution A and 125 ml of solution B were transferred to a clean 1 L glass beaker. A stirrer bar was added, and the solutions were thoroughly mixed while measuring the pH. Small volumes of solution B were added with a micropipette until the pH reached 7.75.

In the final step of the buffer preparation, the following amounts of enzymes were added while stirring continued until everything was dissolved:

1. Trypsin, 24 U/ml (Sigma T9201).
2. Chymotrypsin, 20 U/ml (Sigma C4129).
3. Amylase, 50 U/ml (Megazyme, BLAAM100).
4. Lipase, 4 U/ml (Sigma L3126).
5. Bile, 1 g/L (Sigma B3883).

4.2.4 Intestinal digestibility procedure

The modified Tilley and Terry (1963) assay that was developed by Ross *et al.* (2013) was followed to determine the intestinal digestibility.

Of each oilcake sample, an amount of 0.5 g was weighed out into a labelled 250 ml Nalgene bottle. Magnetic stirrers were inserted into the bottles and each bottle was placed on a magnetic stirrer plate. Rumen fluid (10 ml) was added to all the samples, except for two of the CSND control bottles. An amount of 40 ml of the Goering and Van Soest (1970) buffer was added to each sample, but 50 ml were added to the two CSND control bottles that did not contain rumen fluid. All the Nalgene bottles were gassed with CO_2 and the incubations were done in a temperature-controlled room at 39°C. After 12 hours of incubation (Phase 1), 2 ml of 3M HCl was added to each bottle, followed by 2 ml of the pepsin solution (0.013 M HCl and 0.6 g pepsin per 1 L solution). This initiated Phase 2 of the procedure where incubations were done for one hour, followed by the addition of 2 mL of 2M NaOH to neutralize the solution at a pH of around 5. In Phase 3, 10 ml of the potassium phosphate buffer (1.8M) with a pH of 7.75 was added to all the sample bottles followed by incubation for 24 hours. After the incubation period, 2 ml of 3M HCl were added to each bottle to inhibit further microbial activity. All the bottle contents were transferred to 125 ml plastic cups. Bottles were flushed with distilled water until all the residues were transferred to the cups. Glass microfiber filters with a pore size of 1.5 µm (Whatman 934-AH; Piscataway, New York) were numbered and dried in a 50˚C oven overnight before cooling and weighing them accurately. All the samples were then filtered through the glass microfiber filters under vacuum and dried for 12 hours in a forced draught oven at 100˚C. After drying, samples were cooled in a desiccator and accurately weighed.

4.2.5 Chemical analyses

The dry matter (DM) content of the oilcake meals, maize silage neutral detergent (CSND), positive (freeze dried blood) and negative (burnt blood) controls were done by drying

samples for 24 h at 105°C in a forced draught oven according to the AOAC (2002) Official Method 934.01. Ash was determined according to the AOAC (2002) Official Method 942.05. A Leco FP-528 was used to determine the N content of all the substrates according to the AOAC (2002) Official Method 990.03. Because some of the fine particles, including undigested CP, would have been impregnated in the microfibre filters, the complete filter discs had to be analysed for N. The Leco used for N analysis in the Department's laboratory cannot accommodate such large samples, therefore the filter discs containing the residues were sent to the Analytical Laboratory of the Western Cape Department of Agriculture at Elsenburg for N determination. The filter discs were digested according to the Kjeldahl method and N was read using a Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, Mass, USA).

4.2.6 Statistical analysis

The trial was conducted as a randomized block design; therefore, data were analysed according to a main effects ANOVA using Statistica 10 (2018). Main effects were treatment (Bioprotect® vs distilled water), protein source (soybean, canola or sunflower meal) and block (rumen fluid from six different cows). Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

4.3 Results and discussion

Results of the intestinal digestion trial are presented in Table 4.1.

Table 4.1 The effect of Bioprotect® treatment of three protein sources on total intestinal CP digestion (%), as determined with the Ross assay.

Protein source	Treatment[1]		SEM	*P*
	Bioprotect®	Control		
Soybean OCM[2]	74.7	73.6	0.872	0.378
Canola OCM	62.1	63.9	0.711	0.142
Sunflower OCM	70.53	71.5	0.520	0.239

[1]Treatments: Protein substrates were treated with either Bioprotect® or dH_2O at a rate equivalent to 0.5 L per 1% CP per tonne.
[2]OCM = oil cake meal.
SEM = Standard error of the mean.

No significant differences in intestinal CP digestibility were observed between treatments for any of the oilcake meals. However, when analysing the different oilcakes as main effects, CP ID differed ($P < 0.001$) between all three protein sources. Mean values were 74.2% for soybean meal, 71.0% for sunflower meal and 63.0% for canola meal. Results from the *in sacco* trial (Chapter 3) indicated that Bioprotect® increased effective protein degradation in the rumen, whereas the *in vitro* trial suggested that Bioprotect® decreased CP degradability of canola meal after 8 and 16 h of incubation and tended to decrease degradability of soybean CP after 16 h. The magnitude of the differences in CP degradation between treatments observed from 4 to 16 h in the *in vitro* trial and discussed in Chapter 3, suggested that Bioprotect® may indeed have a depressing effect on CP degradability, but that the effect may be shadowed by an increase in the soluble fraction that had been observed in the Bioprotect® treatment. The current trial was thus done to determine if Bioprotect® might have a post-ruminal effect on undegraded protein, but results indicated that treatment had no effect on the ID (sum of the ruminal, gastric and small intestine digestibility valus) of CP in any of the substrates. An increase in the water solubility of CP was observed after treating all three protein sources with Bioprotect® (Chapter 3). The effect of treatment on the degradation of the soluble protein is, however, unknown and this was the focus of a next trial that will be discussed in Chapter 5.

Results of the current study were consistent with those found in documented studies. In a previous *in vitro* intestinal digestibility study reported by Borucki Castro *et al.* (2007), it was found that different treatments of soybean OCM resulted in similar CP digestibility values as those observed in the current trial. These authors found that expeller, lignosulfonate and heat treatment of soybean meal resulted in disappearance values ranging from 74.3 to 79.4%, whereas in the current study the values were 74.7% (Control) and 73.6% (Bioprotect®). In a study by Paz *et al.* (2014), the intestinal digestibility of canola RUP was reported to be 72.4%.

The variation observed between different studies may occur due to different varieties of soybean and different geographic locations of soybean production. The processing of feedstuffs also affects the RUP digestibility. Small intestinal digestibility of CP was determined by Wang *et al*. (2016) for the same three OCMs that had been used in the current study. These authors used an *in situ* intestinal incubation method and found digestibility values of 98.1% for soybean, 82.6% for sunflower and 88.5% for rapeseed (canola) meal. Borucki Castro *et al.* (2007) did an *in situ* and *in vitro* trial and also found the *in situ* CP digestion values of soybean meal to be high (98.5%). The higher values found in the *in situ trial* could have been caused by the particle loss during intestinal transit or machine water washing of bags. Another reason for the values to differ could have been

caused by hindgut proteolytic bacteria during the total tract *in situ* procedure where bags were retrieved from faeces (Borucki Castro *et al.*, 2007).

Different methods were used in previous studies and some showed similar results when compared with the current study. Although the Ross assay is often used by feed analysis laboratories and results are used in feed formulation programs, not many results have been published where this assay had been used to determine treatment effects on intestinal CP digestibility.

4.4 Conclusion

Bioprotect® (a potential protein binder) treatment of three protein-rich oilcake meals commonly used in dairy cow diets, had no effect on the intestinal CP digestibility. In the previous chapter, results suggested that Bioprotect® treatment might increase RUP values of certain protein sources (e.g. canola meal) at specific incubation times (8 and 16 h), but results were not conclusive. Considering the nett effects of Bioprotect® (*in sacco* and *in vitro*) it appeared that Bioprotect® treatment rather increased apparent ruminal CP degradation. However, regardless of the treatment effect on ruminal CP degradation, positive or negative, the effect was not extended to total intestinal CP digestion as determined by the Ross assay. Because of an increase in the water solubility of CP effected by Bioprotect® treatment in all three protein meals, followed by a lack of treatment response on ID values, it is hypothesised that Bioprotect® treatment might decrease the degradability of soluble protein. This was the focus of the next chapter.

4.5 References

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Chapter 5

The effect of Bioprotect® on buffer soluble protein and degradability of soluble protein

Abstract

This study included two trials and the objectives were to determine the effect of a potential protein binder on buffer soluble protein and then to determine, the degradability of the soluble protein. The protein-rich feedstuffs, commonly included in dairy cow diets, which were used in the soluble protein trial were soybean, sunflower and canola oilcake meals. Each OCM was milled through a 1 mm sieve using a laboratory hammer mill before treating with either Bioprotect® or distilled water (Control). The relevant treatments were applied by spraying an equivalent of 0.5 L per 1% CP per tonne onto the substrates. In the first trial, samples were incubated in a borate-phosphate buffer for 1 hour at 39˚C. Following centrifugation, the supernatants were analysed for N content and soluble protein was calculated. To determine the degradability of soluble protein in the second trial, the same treatment and buffer solubility protocols were followed, but because soybean meal had the greatest buffer solubility in the first trial, it was used as the protein source of choice for the second trial. After the buffer solubility phase, 20 ml of the respective supernatants were added to an incubation medium consisting of 40 ml of Goering-Van Soest buffer and 10 ml of rumen liquid. Four ruminally cannulated lactating Holstein cows were used as rumen liquid donors. Samples were incubated in 100 ml Nalgene bottles in a temperature-controlled room at 39°C for 0, 2, 8 or 24 hours, before they were centrifuged, and the supernatant analysed for N content. Bioprotect® and distilled water treatment of the protein sources resulted in respective protein buffer solubility values, expressed as percentage of CP, of 20.2 and 13.1% for soybean meal ($P < 0.001$), 27.5 and 26.6% for canola meal ($P = 0.475$) and 28.4 and 27.1% for sunflower meal ($P = 0.594$). Because buffer solubility of CP was only increased in the case of soybean meal, it was decided to use only soybean meal to determine the effect of treatment on the degradability of soluble protein. Results showed that Bioprotect® decreased ($P < 0.05$) the degradability of soluble protein. After 2, 8 and 24 h of incubation, protein degradation values were 83.8, 85.4 and 88.4% for the Bioprotect® treatment and 94.3, 98.3 and 99.1% for the Control treatment, respectively. It was concluded that, although Bioprotect® increased the buffer solubility of soybean meal CP, it decreased the in vitro CP degradation thereof. It therefore appears that soluble protein is not

necessarily degraded completely in the rumen and that certain treatments of protein sources may alter the extent of degradation.

5.1 Introduction

Optimization of protein utilisation has become an important factor in ruminant animal diets because of protein being a high cost nutrient. Attention was drawn to the breakdown of feed proteins and microbial protein synthesis in the rumen since the seventies and eighties (Meyer and Van der Walt, 1983). Protein solubility is one of the factors that affect ruminal protein degradation (Stern *et al*., 2006) with soluble protein being fully degraded in the rumen. According to Zayas (1997), information about potential protein utilisation and functionality of feeds can be derived from their solubility values.

Multiple factors affect the solubility of proteins. Environmental factors, such as pH, temperature, ionic strength and processing conditions all play a role (Zayas, 1997). Regarding processing conditions, heat has a significant effect on protein solubility (Sashikala *et al*., 2015). Other factors include molecular weight, composition and sequence of AA, as well as polar and non-polar groups (Zayas, 1997).

Protein solubility plays an important role in the determination of protein degradability (Bach *et al*., 2005) and it is an indicator of degradation when different samples of the same feedstuff are compared (Stern *et al*., 1994). The susceptibility of protein to microbial proteases is determined by the solubility of the protein, which thus affects degradability (Bach *et al*., 2005). The water solubility of all proteins is important for the digestion and absorption of protein (Žilić *et al*., 2006).

Degradation of soluble protein cannot be estimated by the *in sacco* method (Hedqvist *et al*., 2006). A variety of buffers were used in previous studies to measure the solubility of the protein source, aiming to correlate it with the *in sacco* method (Hedqvist *et al*., 2006). Proteins degraded by rumen microorganisms are assumed to be related to the solubility of these proteins with comparable ionic strength and pH in rumen fluid or a mineral buffer (Mahadevan *et al*., 1980).

The objectives of the current study were firstly to determine the effect of Bioprotect® treatment of soybean meal, canola meal and sunflower meal on buffer solubility of protein, and secondly to determine the degradability of soluble soybean protein.

5.2 Materials and methods for protein solubility (Trial 1)

5.2.1 Treatments

Oilcake meals of soybean, sunflower and canola were used in the trial. Each oilcake feed was milled through a 1 mm screen using a laboratory hammer mill (Scientic, RSA). Treatments included the potential protein binder (Bioprotect®) and distilled water (Control). Treatments were applied by spraying the relevant solutions on the substrates at a rate equivalent to 0.5L per 1% CP per tonne.

5.2.2 Preperation

The Nordic Feed Evaluation System (Åkerlind *et al.,* 2011) was used to determine crude protein solubility. In the method, a modified version of the borate-phosphate buffer proposed by Licitra *et al.* (1996) was used. The buffer preparation is indicated in Table 5.1.

Table 5.1 Preparation of the borate-phosphate buffer (NorFor, 2006) used in the trial as modified from Licitra et al. (1996).

Reagents	Quantity
Mono-sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	12.2 g
di-sodium tertraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$)	8.91 g
Distilled water	1 L

The reagents were accurately weighed out and transferred to a 1 L volumetric flask. A volume of 900 ml of distilled water was added and the contents swirled until all the reagents have dissolved, before filling the flask to the 1 L mark with distilled water. The pH of the solution was checked to confirm the required value of 6.75. The buffer solution was then pre-heated to 39˚C before incubation started.

5.2.3 Procedure

An amount of 1.5 g of each protein source was accurately weighed out and transferred to 100 ml screw-top centrifuge tubes before adding 50 ml of the borate-phosphate buffer (39˚C) to each tube. A blank sample (50 ml of the buffer alone) was also included in the incubation series. Samples were stirred with a glass rod, capped and incubated for one hour at 39˚C in a temperature-controlled room. During incubation, the tubes were shaken by hand every 15

min. After incubation, samples were centrifuged at 3000 x g for 10 min, before pipetting 20 ml of the supernatant into 30 ml glass vials. The whole process was repeated three times. Samples were analysed for N in the analytical laboratory of the Western Cape Department of Agriculture at Elsenburg.

5.2.4 Chemical analysis

Dry matter of treated substrates was determined after drying samples at 105°C for 24 h in a forced draught oven (AOAC, 2002: Official Method 934.01). Ash was determined after DM analysis according to the AOAC (2002) Official Method 942.05. In the case of the soluble protein supernatants where no substrate digestion was required, N content of the solution was read directly using a Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, Mass, USA).

5.2.5 Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine the effect of treatment (Bioprotect® vs distilled water) for each protein source separately, using Statistica 10 (2018). Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

5.3 Materials and methods for degradability of soluble CP (Trial 2)

5.3.1 Treatments

Because the buffer solubility of CP only differed between treatments in the case of soybean OCM, it was decided to determine the degradability of soluble protein in soybean OCM only. Milling of the meal, as well as the application of Bioprotect® or distilled water to the substrate, was the same as for Trial 1, described in section 5.2.1.

5.3.2 Preparation

The procedures of collecting rumen liquid and preparation of the Goering and Van Soest (1970) buffer were as previously explained in Chapter 3 (Protocol reference number AUC-2018-6802). Amounts of 1.5 g of the substrate were accurately weighed out in 100 ml Nalgene bottles and a 20 mm magnetic stirrer was added to each bottle. Buffer soluble protein was prepared in the same way as in Trial 1 (Table 5.1).

5.3.3 Procedure

Only two methods were found in the literature to determine the degradation rate of soluble protein (Hedqvist and Udén, 2006; Crossland *et al.* 2012). The method of Crossland *et al.* (2012) is elaborate and, according to the authors, it has not been validated. The method

described by Hedqvist and Udén (2006) was modified by the supervisor of the current study by using borate-phosphate buffer (as used in Trial 1) to solubilise the protein instead of McDougall's buffer. Another modification was to use the Goering and Van Soest (1970) buffer in the incubation medium instead of the one described by Hedqvist and Udén (2006). A reagent blank, as well as a rumen fluid blank, were included in the current trial, while Hedqvist and Udén (2006) only included rumen fluid blanks in their method. The method used in the current trial has not been used before and should be tested in future validation studies as well.

After preparing the samples, 40 ml of the Goering and Van Soest (1970) buffer and 10 ml of rumen liquid were added to each Nalgene bottle. Rumen liquid was collected from four lactating and ruminally cannulated Holstein cows, resulting in four sets of incubation preparations per treatment. In addition to the above, 20 ml of either the soluble protein supernatant or the borate-phosphate buffer was accurately pipetted into the bottles. The supernatants were obtained after treating substrates with either Bioprotect® or distilled water. A control group (rumen liquid blank) that did not contain the oil cake substrate, was included for each cow and incubation time, containing rumen liquid, Goering and Van Soest (1970) buffer and borate-phosphate buffer. Bottles were subsequently gassed with CO_2 and placed on stirrer plates in a temperature-controlled room with the temperature set at 39°C. Incubation times were 0, 2, 8 and 24 h. The 0 hours did not contain any rumen liquid, but only Goering and Van Soest (1970) buffer and supernatant and was used as a reagent control. To stop the fermentation after each incubation time, the bottles were placed in ice water for 15 minutes, where after they were centrifuged at 3000 x g for 10 minutes. Aliquots of 20 ml of the supernatant were transferred to 30 ml screw top test tubes and submitted to the analytical laboratory of the Western Cape Department of Agriculture at Elsenburg for N analysis. The rumen liquid blank was used to correct for microbial protein.

5.3.4 Chemical analysis

The soybean oil cake was analysed according to AOAC (2002) methods for DM (Official Method 934.01) and ash (Official Method 942.05). For CP analyses of the oil cakes, a Leco FP-528 was used to determine N content according to the AOAC Official Method 990.03. In the case of the soluble protein supernatants where no substrate digestion was required, N content of the post-incubation supernatants was read directly using a Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, Mass, USA).

5.3.5 Statistical analysis

Protein disappearance data per time were subjected to a main effects ANOVA using Statistica 10 (2018). Main effects were treatment (Bioprotect® vs distilled water) and cow (rumen liquid was collected from different cows). Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.1$.

5.4 Results and discussion

5.4.1 Protein solubility

Results of the buffer solubility of protein in the different oil cakes are presented in Table 5.2.

Protein solubility is expressed as % of substrate DM, as well as % of CP. Treatment only affected buffer solubility ($P < 0.001$) in the case of soybean meal, where Bioprotect® increased soluble CP as % of substrate DM with 44% and soluble CP as % of substrate CP with 54%. This is contrary to what has been observed in the *in sacco* trial (Chapter 3) where fwater solubility of CP was increased by Bioprotect® in all three oil cakes. Griffiths (2004) also observed significant differences between water solubility and borate-phospahte buffer solubility of various protein sources, including soybean, sunflower and canola oil cakes. However, water solubility cannot necessarily be compared with solubility in a borate-phosphate buffer. According to Lee *et al.* (2003), the solubility of soybean protein in different solutions depend on the pH of the solution, the ionic strength of the salts in the solution, and temperature of the solution.

Table 5.2 The effect of Bioprotect® treatment of different protein oil cakes on the solubility of protein in a phosphate-borate buffer.

Oilcake meal	Treatment		SEM	*P*
	Control	Bioprotect®		
Soybean				
Soluble protein, % of DM	5.7	8.2	0.098	<0.001
Soluble CP, % of CP	13.1	20.2	0.642	<0.001
Sunflower				
Soluble protein, % of DM	11.7	11.5	0.344	0.683
Soluble CP, % of CP	27.1	28.4	1.718	0.594
Canola				
Soluble protein, % of DM	11.6	11.2	0.178	0.147
Soluble CP, % of CP	26.6	27.5	0.808	0.475

Heat treatment of the protein source (e.g. roasting and extrusion of the oil cake) also has a significant effect on the protein solubility. According to Mosimanyana and Mowat (1992), heat treatment of protein sources caused a decrease in the amount of rapidly soluble N, as well as in the rate and extent of CP degradation. The amount of slowly degradable CP, however, increased. Similar observations were made by Griffiths (2004) where extrusion decreased both water and buffer solubility of various protein sources.

In the current study, buffer soluble CP values were lower than those reported by Nel (2012). In that study, where the same borate-phosphate buffer was used, soluble CP values of 25.5% and 42.3% were reported for soybean and sunflower oil cakes, respectively. For the same protein sources, Griffiths (2004) reported soluble CP values of 29% and 26%, respectively. In the latter case, the value for sunflower protein agreed with that of the current study. Macgregor *et al.* (1978) used solvent extracted and dehulled soybean meal and reported a protein solubility of 22.4%.

Differences in documented CP solubility values are not only the result of the physical or chemical characteristics of the specific protein source. Inter-laboratory differences have been observed by Nel (2012) where significant differences in the borate-phosphate solubility of various protein sources were obtained on split samples by two independent laboratories.

Because soybean oil cake had the highest soluble protein values in the current study, and also because the values were higher for the Bioprotect® treatment than for the dH_2O treatment, it was decided to determine the degradability of soluble protein only for soybean oil cake.

5.4.2 Degradation of soluble protein

Results of the treatment effects on soluble protein degradability of soybean meal is presented in Table 5.3 and Figure 5.1.

Table 5.3 Effect of Bioprotect® treatment[1] on the degradability of soluble protein (%).

Incubation time	Bioprotect®	Control	SEM[2]	*P*
0 h	20.2	13.1	0.642	< 0.001
2 h	83.8	94.3	1.679	0.022
8 h	85.4	98.3	1.122	0.004
24 h	88.4	99.1	0.400	< 0.001

[1]Substrates were either treated with Bioprotect® or dH_2O by spraying at a rate equivalent to 0.5 L per 1% CP per tonne.
[2]SEM = Standard error of the mean.

Table 5.3 indicates that Bioprotect® significantly increased the solubility of CP (0 h), which has been discussed above, but when the soluble protein was exposed to rumen liquid, Bioprotect® significantly decreased the degradability of soluble CP over time. The magnitude of the decrease effected by treatment was 11.1% after 2 h, 13.1% after 8 h and 10.8% after 24 h of incubation.

Not all soluble protein, soluble oligopeptides, or soluble amino acids are hydrolysed to ammonia in the rumen. Some escape ruminal degradation but under *in vitro* conditions they cannot be removed from the incubation vessel. Reynal *et al.* (2007) reported that, on average across diets, 27, 75, and 93% of soluble amino acids in soluble protein (>10 kDa), oligopeptides (3 to 10 kDa), and small peptides plus free amino acids (< 3 kDa) that escaped the rumen were of dietary origin. Hence, more ammonia can be produced *in vitro* than *in vivo*.

Mahadevan (1980) concluded that the solubility or insolubility of a protein per sé does not necessarily imply that the protein is resistant to ruminal hydrolysis by bacterial proteases, but that crosslinking disulfide bonds determine resistance to degradation.

Hedqvist and Udén (2006) found a significant variation in degradation rates of soluble protein between feeds, confirming that not all soluble protein is degraded in the rumen. They incubated soluble protein samples for 0, 20, 40, 60, 80, 100, 120, 160, 200 and 240 minutes and reported k_d values (h^{-1}) between 0.33 for red clover to 1.00 for casein. No documented results were found regarding the effect of specific treatments on soluble protein degradation.

The effect of Bioprotect® on soluble protein degradation at various time intervals obtained in the current study is presented in Figure 5.1.

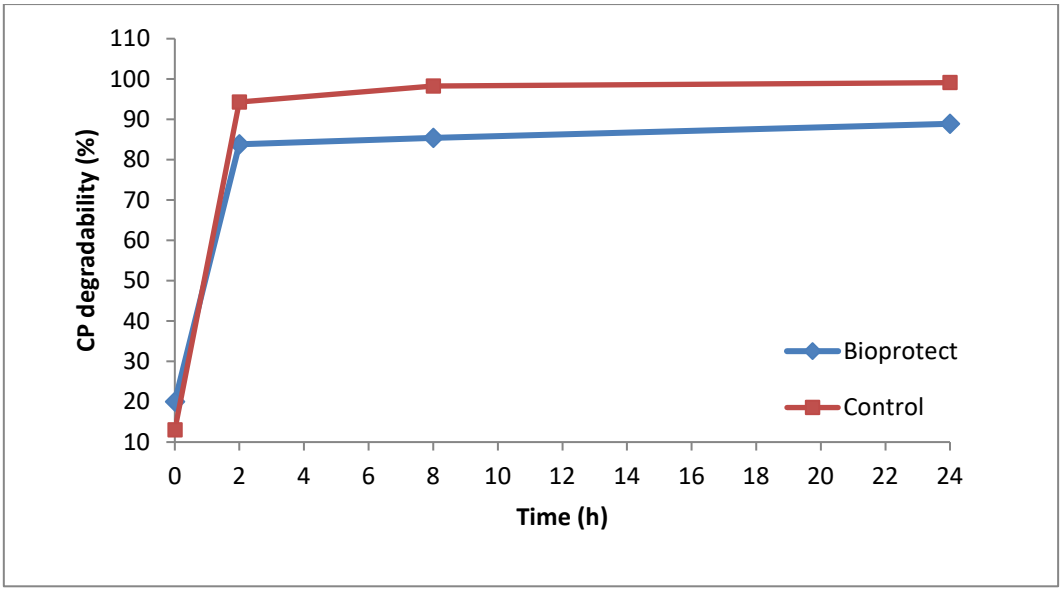

Figure 5.1 Effect of Bioprotect® treatment on the degradability of soluble protein (%).

In Figure 5.1 it can be seen that degradation during the first two hours was significant for both treatments. Mean k_d values of 0.32/h^{-1} and 0.41/h^{-1} were calculated for the Bioprotect® and Control treatments during this time and it is apparent that the Bioprotect® treatment had an immediate effect on the rate of degradation. From 2 to 8 h, mean k_d values were 0.003/h^{-1} for the Bioprotect® treatment and 0.007/h^{-1} for Control. Between 8 and 24 h, the respective k_d values were 0.002/h^{-1} and 0.0005/h^{-1}. Except for the period between 8 and 24 h, k_d values were much lower for Bioprotect® than for Control. Between 8 and 24 h, the Control treatment approached 100% degradation, hence the extremely low k_d value. Over the entire 0 – 24 h incubation period, the mean degradation rate was 0.028/h^{-1} for the Bioprotect® treatment and 0.036/h^{-1} for the Control treatment, clearly demonstrating the depressing effect of the Bioprotect®.

5.5 Conclusion

In the previous chapters it has been shown that the treatment of soybean, canola and sunflower oil cakes with Bioprotect® resulted in increased CP degradation and increased CP solubility compared to the Control treatment (dH_2O). However, although Bioprotect® treatment of soybean OCM again resulted in higher protein solubility compared to the Control, the degradability of the soluble protein was significantly lower than that of the Control treatment. The manufacturers of Bioprotect® claim that the product binds to the amino groups of proteins, thus slowing the degradation rate and increasing the RUP value of vegetable protein sources. In all the trials discussed in the current study, the only suppressing effect of Bioprotect® on protein degradability was observed in soluble protein. The manufacturers also claim that by using Bioprotect®, the dietary CP content of lactating

dairy cow feeds can be lowered by one or two percentage units without compromising milk production. If the soluble protein content of protein sources is high enough and the depressing effect of Bioprotect® on soluble protein degradation in the rumen is significant enough, the nett effect might be carried through to milk responses, but this hypothesis should be tested in a future milk production trials.

5.6 References

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Chapter 6

General Conclusion

According to the suppliers of the protein binder, Bioprotect® slows the rate of ruminal protein degradation because it binds to the amino groups of proteins. The objectives of this study were thus to determine the effect of the potential protein binder on *in sacco* and *in vitro* protein disappearance parameters, protein solubility, degradability of soluble protein and intestinal protein digestibility. Soybean, sunflower and canola oilcake meals were the three protein feedstuffs used in the trials. These feedstuffs are commonly included as protein sources in dairy cattle diets in South Africa.

Formulating dairy cattle diets to meet protein requirements has shifted from formulating for CP alone to that of metabolisable protein which is digested and absorbed as AA in the small intestine. The MP is derived from RUP, microbial protein and endogenous protein, but there needs to be a fine balance between RUP and RDP for optimal microbial protein synthesis and animal production. Optimising diet formulation would also result in more space for other raw materials. From the literature, it appears that there is a need for research on ways to treat protein sources in order to decrease ruminal protein degradability of oil cake meals and the effect thereof on intestinal digestibility.

Bioprotect® increased the a-value (0 h incubation) of all the substrates, indicating a higher water solubility following treatment. In the *in sacco* trial, Bioprotect® did not lower DM or CP degradability for any of the substrates. As a result of the higher a-values obtained with Bioprotect® treatment, the effective CP degradability (eDegCP) of all the substrates increased. In the *in vitro* trial, treatment * time interactions showed that Bioprotect® decreased CP degradation in canola meal after 8 and 16 h of incubation and tended to decrease 16 h CP degradability in soybean meal. It was concluded that Bioprotect® appears to increase CP solubility, but the effect of treatment on CP degradability was not conclusive due to different tendencies observed in the *in sacco* and *in vitro* trials. However, the difference between treatments in the magnitude of *in vitro* CP degradability observed from 4 to 16 h suggested that Bioprotect® may indeed have a depressing effect on the degradability of the potentially degradable fraction but this effect may be shadowed by the increase in the soluble fraction observed in the Bioprotect® treatment. Due to the significant impact of the soluble fraction on the calculation of effective degradability, the result was a

higher effective CP degradability observed in the Bioprotect® treatments. The CP degradability of the soluble protein fraction was unknown and was consequently investigated in the last part of this study.

The effect of Bioprotect® treatment on the intestinal protein digestibility of the three oil cakes was the focus of the second phase of the study. The Ross assay was used in this trial, but results showed that treatment had no effect on the intestinal CP digestibility of any of the three protein sources. Although results of the *in vitro* trial suggested that Bioprotect® treatment might increase RUP values of certain protein sources (e.g. canola meal) at specific incubation times (8 and 16 h), the nett effects of the *in sacco* and *in vitro* trials indicated that Bioprotect® treatment rather increased apparent ruminal CP degradation. However, regardless of the treatment effect on ruminal CP degradation, positive or negative, the effect was not extended to total intestinal CP digestion as determined by the Ross assay. Because of an increase in the water solubility of CP effected by Bioprotect® treatment in all three protein meals, followed by a lack of treatment response on ID values, it was hypothesised that Bioprotect® treatment might decrease the degradability of soluble protein.

The focus of the last trial was firstly to investigate the effect of Bioprotect® on CP solubility of the three oil cake meals in a borate-phosphate buffer. Although the a-values of the *in vitro* and *in sacco* trials showed that water washing after Bioprotect® treatment increased CP solubility of all three oil cakes, it appeared that treatment only had an effect on buffer solubility of CP in the case of soybean oil cake. Therefore, only soybean oil cake was used to investigate the effect of Bioprotect® on the degradability of soluble CP. Whereas the buffer solubility of CP was increased following Bioprotect® treatment, the degradability of the soluble protein was significantly lower than that of the Control treatment. The manufacturers of Bioprotect® claim that the product binds to the amino groups of proteins, thus slowing the degradation rate and increasing the RUP value of vegetable protein sources. In all the trials done in the current study, the only suppressing effect of Bioprotect® on protein degradability was observed in soluble protein. The manufacturers also claim that by using Bioprotect®, the dietary CP content of lactating dairy cow feeds can be lowered by one or two percentage units without compromising milk production. If the soluble protein content of protein sources is high enough and the depressing effect of Bioprotect® on soluble protein degradation in the rumen is significant enough, the nett effect might be carried through to milk responses, but this hypothesis should be tested in future milk production trials.